On the use of titanium based materials as non-resorbable bone substitutes

Doctoral thesis by

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holder or the unit which grants the doctorate.

To my parents, to my wife Victoria
and especially to my children
Oscar, Edvard and Caroline.

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Ladegaardsøen, 25th of August, 2011

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List of papers

The following papers are submitted in partial fulfillment of the requirements for the Degree of Philosophiae Doctor at the faculty of Dentistry, University of Oslo, Norway:

I. Wohlfahrt JC, Monjo M, Ronold HJ, Aass AM, Ellingsen JE, Lyngstadaas SP. Porous titanium granules promote bone healing and growth in rabbit tibia peri-implant osseous defects. *Clin Oral Implants Res* 2010;21:165-173.

II. Wohlfahrt JC, Aass AM, Ronold HJ, Heijl L, Haugen HJ, Lyngstadaas SP. MicroCT and Histological Analysis of Animal Experimental Degree II Furcation Defects Treated With Porous Titanium Granules or Deproteinized Bovine Bone. *J Periodontol* 2011;Jun 21.[Epub ahead of print]

III. Wohlfahrt JC, Aass AM, Ronold HJ, Lyngstadaas SP. MicroCT and Human Histological Analysis of a Peri-Implant Osseous Defect Grafted with Porous Titanium Granules: A Case Report. *Int J Oral Maxillofac Implants* 2011;26:e9-e14.

IV. Wohlfahrt JC, Lyngstadaas SP, Rønold HJ, Saxegaard E, Ellingsen JE, Karlsson S, Aass AM. Porous Titanium Granules in the Surgical Treatment of Peri-Implant Osseous Defects – A Randomized Clinical Trial. *Int J Oral Maxillofac Implants* 2011;Accepted for publication.

V. Wohlfahrt JC, Lyngstadaas SP, Heijl L, Aass AM. Porous Titanium Granules in the Treatment of Mandibular Degree II Furcation Defects- A Consecutive Case Series. *J Periodontol* 2011;May 12.[Epub ahead of print]

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Abbreviations commonly used in this thesis

BoP =bleeding on probing

BMU =basic multicellular unit

CAL =clinical attachment level

c.p. =commercially-pure (titanium)

DBBM =deproteinized bovine bone mineral

EDTA =ethylenediaminetetraacetic acid

GI =gingival index

GR =gingival recession

GTR =guided tissue regeneration

HAp =hydroxylapatite

HTX =hematoxylin

ISQ =implant stability quotient

L.M. =light microscope

microCT =micro-computed tomography

n =number (i.e., number of individuals in sample population)

n.s. =non significant

O.M. =original magnification

OFD =open flap debridement

PI =plaque index

PD =pocket depth

PDL =periodontal ligament

PTG =porous titanium granules

PPD =probing pocket depth

RFA =resonance frequency analysis

TiO_2 =titanium dioxide

WPTG =white porous titanium granules i.e., PTG heat oxidized at 900 °C.

Introduction

The clinical challenge

Throughout the adult life, bone is constantly adapting to stress by remodeling, and challenged by various insults from trauma, infection, pathological processes and aging. In the clinic this often present itself as loss of mineralized tissue resulting in impaired form and function. When a clinical intervention is necessary to restore lost bone and re-establish or maintain quality of life for the patients the therapy often involves the use of bone replacement materials. The clinician is then confronted with several clinical challenges, both related to the patient per se, as well as regarding the mechanical and chemical properties of the biomaterial used. This thesis will focus on titanium used as a non-resorbable bone substitute material in peri-implant and periodontal osseous defects.

Some patient related factors:

1) Systemic and local medical conditions
2) Medications
3) Social factors (e.g., smoking)
4) Psychosocial factors (e.g., stress)
5) Compliance (e.g., infection control)

Some material related factors:

1) Biocompatibility
2) Immunogenicity
3) Mechanical strength
4) Availability
5) Applicability

Titanium from a historical perspective

In 1790, an English cleric, Reverend William Gregor, discovered a new element in a sample of sand from Tregonwell Mill in Cornwall, which he decided to call “menaccanite.” Five years later a German chemist, Martin Heinrich Klaproth, discovered the exact same element but instead named it “titanium”, or “titankalk.” Even if Klaproth recognized that Gregor was the first discoverer of the novel element, titanium became the acknowledged name.[1] It took the scientific community another 155 years to discover that titanium is accepted by biological tissues and therefore exceedingly useful in medicine.[2, 3]

Titanium from a general material perspective

Titanium is the fourth most frequent metal on planet Earth and is only exceeded by aluminum, iron and magnesium.[4] Titanium (Ti, atomic nr 22 and atomic weight of 47.9) is a so-called transition metal and exists in a number of crystalline forms; it is thus defined as an allotropic element.[4] In room temperature, titanium exists as a hexagonal close-packed crystal structure known as the alpha phase. When heat treated above 883ºC, titanium transforms from the alpha phase to a cubic structure called the beta phase.[4] The properties of titanium changes dramatically when transformed from alpha to beta phase which is interesting from the perspective of the medical performance.[4] Due to its high affinity for oxygen, titanium exists mostly in nature as titanium dioxide (TiO_2) in many forms such as rutile (TiO_2), ilmenite (titanium iron oxide ($FeTiO_3$)), anatase (another form of TiO_2), brookite and sphene (titanite).[5] Rutile is the most attractive and common ore for extracting titanium and contains 95% TiO_2 followed by ilmenite with 50-65% TiO_2.[4] These minerals consequently have the same chemistry (i.e., TiO_2) but they are different in structure. Quite soon after titanium was discovered it turned out to be difficult to isolate, hence its expensive nature. 99.9% pure titanium was first isolated by the chemist Matthew A. Hunter in 1910 with the so-called Hunter process.[1] In 1936, William J. Kroll invented a more efficient method consequently named the Kroll process, which since then has been the "modus operandi" for producing commercially-pure (c.p.) titanium.[4] The Kroll process can be methodologically described as conversion of TiO_2 with chlorine to $TiCl_4$ which is then reduced with magnesium to Ti and $MgCl_2$. The end result is titanium of 99.3% purity.[5]

American Society for Testing and Materials (ASTM) defines titanium as grade 1-31, with grade 1-4 being c.p. unalloyed titanium. The four unalloyed c.p. titanium grades (TiCP 1-4 (Alpha) F67, Grade 1—UNS R50250; Grade 2—UNS R50400; Grade 3—UNS R50550; and Grade 4—UNS R50700) are defined based on the amount of impurities (a.k.a. interstitial

elements), which affects the tensile and strength properties of the material. The contents of C, N and H are similar in all four grades, while the amount of Fe and O increase to some extent with the grades.[6]

C.p. titanium is as strong as many types of steel but about 45% lighter.[7] Titanium is a reactive metal which means that in air, water, or as a matter of fact in any electrolyte, an oxide is spontaneously formed on its surface.[8] Already after 10 nanoseconds a mono-atomic layer of oxide is formed on the titanium surface.[9] The oxide layer is initially very thin and has been described to be only three to six nanometer in thickness.[10] It is also shown that the growth of the oxide layer can be accelerated by an increase in temperature.[9] Importantly this oxide layer is one of the most resistant minerals known and is hence protective against chemical attack.[8] The oxide layer also makes titanium passive, which means that it behaves more like a noble metal due to the stable, corrosion-resistant oxide layer.[11, 12] This also implies a low rate of diffusion of metal ions into the surroundings (i.e., the biological tissues.[13]) The oxide layer will spontaneously and instantaneously repair itself if damaged, which for example might occur during seating of a titanium implant in bone.[12] It has also been speculated that another advantage with TiO_2 from the perspective of bonding between bone and the implant surface is the high dielectric constant,[9] which can be defined as relative (i.e., relative to vacuum) electric permittivity (i.e., electric resistance) or a measure of how a material is affected (i.e., the ability to polarize the electric charges) and affects (i.e., the ability to transmit) an electric field. The reference value for vacuum is one. The dielectric constant for anatase is 48, for brookite 78, and for rutile 110-117, which all are significantly higher than for any other metal oxides.[13] In comparison the dielectric constant for iron oxide is 14.2 and for copper oxide 18.2, whereas for water the dielectric constant at normal body temperature is approximately 74 and at a room temperature of 20 °C it is 80. At the least hypothetically this would result in stronger van der Waal´s bonds between the TiO_2 surface and water as compared to other oxides.[9, 13]

Thus, the benefit of titanium as an implant material is the combination of a light and mechanically strong nucleous (i.e., the bulk metal) and a chemically very attractive thin surface coating (i.e., the TiO_2 layer).[9] These properties, as well as the repeatedly proven fact that biological tissues accept it, make titanium interesting as a biomaterial.

Titanium from a biomaterial perspective

The potential of an element to be implantable, accepted by biological tissues, and remain in function as a load bearing device has gained a lot of interest through the history of medicine and replacement therapy. Within the field of dentistry, a wide plethora of attempts have been made, through the years, to replace lost teeth with crowns retained by various types of implanted devices, anchored to the jawbone. Archeological findings from China, Egypt and South America show that ancient dental implants were made of stone or ivory.[14] An implant made of a tooth shaped shell dating back to 600 A.D. was excavated by a Mayan indian archeological expedition in Honduras led by Dr. Wilson Popenoe in the 1931. The first metal tooth implant probably dates back to late year 100 or early year 200 A.D. and was discovered in a scull found in an archeological excavation of a Gallo-Roman population in Chantambre in France. This implant, made of iron, had replaced a second upper premolar and notable was the close connection between bone and implant, with seemingly no inter-positioned fibrous tissue.[15] Implants made of gold or ivory were used in the 16th and 17th century, whereas allogenic (i.e., from one patient to another) tooth implants were popular in the 17th century England, France and North America.[14] In the late 19th and early 20th century, a change was made towards using metal implants made of e.g., lead, iridium, gold, tantalum, stainless steel, silver or cobalt alloy.[14, 16] Longevity of these early implants was marginal and usually not more than a few years prior to loss.[14, 16] In 1913 Dr. E. J. Greenfield gave an oral presentation at the monthly meeting of Academy of Stomatology of Philadelphia where he reported on the

utilization of hollow and latticed cylinder implants made by iridio-platinum wires soldered together with 24 carat gold and placed in trephine sockets filled with bismuth paste. Greenfield had developed his novel technique based upon the surgical ligation of bone fractures by silver-wire sutures as he had seen performed by orthopedic surgeons. He was extremely confident in his reporting and when asked: "How long will this platinum root last?" he had stated: "I do not expect to live long enough to answer that question."[17] To the best of knowledge no long-term follow-up of Greenfield´s implants was ever published, but it is still noteworthy that much of the pioneering techniques, described by Greenfield, are relatively similar to techniques used in implant dentistry of today.

In 1924, Zierold[18] conducted an animal experimental study in 63 dogs with the aim to evaluate tissue reactions from a wide range of metals surgically implanted in vital bone. The metals were gold, zinc, copper, nickel, aluminum bronze, aluminum, magnesium, silver, high and low carbon steel, iron lead and stellite. The dogs were euthanized at 2 or 6 weeks after implantation, and sections with bone and the metallic implants were analyzed by histology. Zierold´s analyses showed that none of the implanted metals had fused with bone, but there was a marked difference in tissue reactions to the different metals. Gold, aluminum and stellite seemed to be best tolerated and were all, quote: "surrounded by a dense zone of connective tissue of three or four layers, closely approximated by new bone." He also stated that silver and lead provoked more connective tissue response which he suggested to be an effect of their corrosiveness. Zink, nickel and magnesium seemed to interfere with bone growth. Copper provoked pronounced bone growth but also seemed to be toxic when in close contact with bone. Steel, but also iron, inhibited bone growth, and steel was by Zierold judged to be quote: "least suitable of all for bone prosthesis." Noteworthy is the fact that Zierold considered the corrosive properties of a metal to be of crucial importance for its inert nature. Experimental studies by Venable, Stuck and Beach[19] further elucidated on Zierold´s findings

with regard to corrosiveness and inertness and performed experiments in dogs where pegs of various metals were implanted and the "electrolytic action" (i.e., difference in electrochemical potential) and bone reactions were examined by performing biochemical analyses of the tissues adjacent or adhering to the implanted pegs to study if ions of one metal had been carried to the other. They suggested that the minimal electrolytic activity, and thus low rate of corrosion of the alloy Vitallium (i.e., an alloy of 60% cobalt, 20% chromium and 5% molybdenum and some additional substances), was the reason for the total absence of tissue reactions or bone changes around these pegs. This made Vitallium a potential candidate for use as a surgical implant.

In a report from 1939 Strock[20] presented findings both from treating patients with Vitallium implants as well as from an animal experimental study in dogs. Based on the findings by Venable, Stuck and Beach[19], Strock implanted screws of Vitallium in human extraction sites and reported that in two of three cases the screw was stable, and in one of the cases bone had filled the extraction alveoli in close connection with the implant when evaluated eight months after implantation. As reported by Shulman and Driskel[14] who later followed up on Struck´s patients, at least one implant remained stable and asymptomatic for 16 years i.e., until 1955 when the patient passed away. This probably makes it the very first implant with long term documented success.[14] Bone fill and stable implants after six months of follow-up were also seen in two dogs treated with Vitallium implants as reported by Strock.[20]

In 1940, Bothe, Beaton and Davenport[3] performed an animal experimental study in cats with the aim to compare tissue reactions of various metallic implants. The authors state that: "titanium was fully as well tolerated as Vitallium and stainless steel, perhaps better in that the bone had a tendency to grow into contact with it." Opposite to Veneable, Stuck and Beach[19] they reported that bone reactions were not correlated to the electric potential (i.e., electro negativity) of a given metal. In retrospect one may comment this finding is not surprising

since titanium is pacified by the surface oxide layer and thus do not corrode. In 1951, Leventhal[21] performed animal experimental studies in rabbits and rats to study soft tissue and bone reactions to titanium. The author reported no signs of soft tissue reactions to titanium bars subcutaneously placed at the dorsal aspect of rabbits and that screws inserted in rat femurs were progressively more difficult to remove at six, 12 and 16 weeks. Furthermore, microscopic examination of the bone at the implanted sites revealed no reactions to the titanium screws. Animal experimental studies by Laing, Fergusson and Hodge[22] compared tissue reactions from different metals implanted for six months in muscle of 430 rabbits. 1500 muscle specimens with implanted material were analyzed by histology. The authors report that ”the tissue reaction to titanium and titanium-alloy implants was remarkable for its consistency and its thinness.” Noteworthy in this report is also that zirconium implants were reported to show pronounced tissue reactions and quote: “very unsatisfactory results.” The authors state that “the results of this suggest that titanium and its alloys present enough evidence of their suitability for the manufacture of surgical implants to warrant their continued use in human subjects.”

In 1959 Beder and Ploger[23] presented findings from intraoral usage of titanium bolt and nut-retained plates for stabilization of ostectomized mandibles in dogs, as well as for permanent closure of an oro-antral defect surgically created in the palate. The authors report that in one of the three dogs which had no exposure of the titanium devices during the healing period, complete acceptance of the material was found. This was the first published scientific report documenting intraoral implantation of titanium devices.

In 1969 Brånemark and Breine[24] presented some findings on the performance of titanium after studies in animal experimental models. A microscopic chamber technique had been used to study the function of nutritive capillaries in bone marrow of rabbits and dogs. The chambers, made of titanium, were inserted through the covering tissues and penetrated through bone and

then left in place for up to 150 days. It was reported that the appliances had caused quote: “no undesired side effects on the soft tissue” and that “it was possible to secure a firm anchorage of the titanium appliance in the bone.” These early findings led this group to further explore titanium as a material for surgical implants. In 1969 the Brånemark research group[25] presented findings from an animal experimental study on screw-shaped titanium implants for permanent bone tissue anchorage of dental prostheses. 67 screw-shaped implants had been inserted in dog jaw bone and left for submerged healing for 6-8 weeks. Thereafter second stage surgery was performed, and two weeks later prostheses were manufactured and anchored to the implants. All implants were subjected to a torque resistance test, and a number of the implants were subjected to tensile strength test. Implants were removed en bloc and histological sections prepared. It was suggested that screw-shaped implants made of titanium, if placed with minimized operative trauma, if allowed to heal without communication to the oral cavity and if kept free from gingivitis, become stable, well integrated in bone and with surrounding connective tissues absent of inflammation.

In this context it may be interesting to mention that the first orthopedic hip stem prostheses were developed in the early 1960s by Charnley.[26] Noteworthy is the fact that still today the absolute majority of such orthopedic hip stem implants are manufactured in stainless steel and not titanium. Presumably this relates to the complexity of manufacturing such prostheses in titanium, since the method of turning the material to appropriate shape cannot be used, but instead they have to be casted, which is a much more costly technique. The scope of this thesis is however titanium when used as implant material in the jaws and further discussion on orthopedic implants will thus be left aside.

In 1976 Schroeder, Pohler and Sutter[27] reported findings from an animal experimental study in monkeys on the performance of different types of titanium hollow cylinder implants

sprayed with powdered titanium. These authors also reported that the titanium implant had a quote: “solid incorporation” in bone as seen on radiographs and “an immediate growing in of the bone into the rough implant surface” as seen by both light microscopy and electron microscopy. Schroeder used the expression “functional ankylosis” to describe the solid fixation and incorporation of an implant to mineralized bone.

A great number of other dental implant designs have been suggested and manufactured throughout the years. Examples include the endosseous Linkow blade-vent implant,[28] subperiosteal implants[29] and transosseous implants, such as the mandibular staple bone plate as described by Small.[30] Noteworthy, the first subperiosteal implant was placed and patented by Dr. G Dahl in Sweden in 1941[31, 32] later followed by Goldberg and Gershkoff in 1949[32, 33] in the Unites States who reported on clinical cases with mandibular subperiosteal implants. It is however beyond the scope of this thesis introduction to discuss such implant designs, but instead to discuss implantology from the perspective of titanium as a biomaterial.

In 1977 Brånemark and co-workers[34] published data from up to 10 year follow-up of 211 completely edentulous patients consecutively treated with screw-shaped implant fixtures made of titanium. The fixtures had healed immobilized and quote: “for a long period of time (minimum three months) separated from the oral cavity by the mucoperiosteum.” The fixtures were made of quote: “the purest kind of titanium available” which was ATi 24; 99.6% Titanium (Fe 0.20, 0_2 0.10, N_2 0.04 C 0.05 H20.012 manufactured by Avesta Jernverk. Sweden). The fixtures (XENODENT®) were machined (“turned”) to standardized dimensions. A comprehensive report of the clinical and radiographic, 10 year follow-up data, was presented. Furthermore biopsies from 60 patients had been analyzed by histology. Some previous histological reports on various metals implanted in bone had described a border zone of connective tissue characterized as a “pseudoperidontium.” This was the first clinical study presenting human histological evidence for true “osseointegration”, which was carefully

described in the report. The patients had observation times between nine months and 10 years, and it was reported that: "94% of the upper jaws and 100% of the lower jaws had a stable bridge on osseointegrated implants."

In 1981 Adell et al.[35] reported retrospective data on 1997 titanium dental implants placed in 284 consecutive patients between the years 1965 and 1980.The report was divided into both the development phase and later routine phases of fixture installation and anchorage of prostheses according to the "Brånemark method." After three years of follow-up, persisting fixtures were reported to be between 53% and 91% depending on jaw and routine or development phase of treatment. With regards to bone loss it was also shown that more bone was lost during the first year after installation of the implants (0.8-1.5 mm) and thereafter less pronounced (0.1 mm/ yearly) for the first five to nine years of follow-up. The early findings by Brånemark and co-workers initiated an era with a great number of scientific reports on the clinical use of so-called endo-osseous screw-shaped titanium dental implants.

Biocompatibility

Biocompatibility by principle means "harmonious with life"[36] and is pivotal when implanting a medical device in living tissue with the objective of acceptable long term function. It defines the ability of a specific material to "function in a specific application in the presence of an appropriate host response."[37] The definition also implies that the specific material must be free of any safety concerns for the recipient patient.[37] In 1987, Williams[38] proposed the following definition of biocompatibility: "the ability of a material to perform with an appropriate host response in a specific situation." The major thought behind this definition was that a biocompatible biomaterial has to perform some kind of function i.e., at the least be compatible with the function but preferably actively support the function. [39] It is, though, obvious that even if the biomaterial supposedly shall perform a function, it shall still not harm

the patient. More recently this led Williams[39] to propose a new definition of biocompatibility as follows: "Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy." A noteworthy fact is that, over some time, no implantable biomaterial has so far been shown to be completely free of adverse reactions in humans or animals.[4] Whether this relates to the biomaterial, per se, or instead to host related factors is an issue for discussion.

To evaluate the biological behavior and potential biocompatibility of a novel biomaterial, it will be fundamental to test it from many different perspectives such as cytotoxicity, histocomptibility, mutagenicity and microbiological effects.[37] Such tests are complex and when considering a potential novel application for a specific biomaterial it is of great value that the material has already been successfully used for biological applications. This is obviously one of the crucial advantages with titanium when utilized within the field of osseous reconstructive therapies.

Bone characteristics

Bone is a mineralized connective tissue with specialized functions and consists of 33% organic matrix i.e., 28% type I collagen, and 5% non collagenous proteins. The non-collagenous proteins include osteonectin, osteocalcin, bone morphogenetic proteins, bone proteoglycan and bone sialoprotein. The remaining 67% of bone is inorganic and consists of calciumphosfate in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) crystals.

Microscopically, four types of bone tissue can be defined: woven, composite, lamellar and bundle. Woven bone is soft, unorganized immature bone which forms early during healing and later is replaced with the more mature and organized lamellar bone. The bone tissue seen

in the transitional phase between woven and lamellar bone is known as composite bone. Bundle bone lines the bone adjoining joints and ligaments and is highly perforated (a.k.a. the cribriform plate) since it transmits nerves and vessels and provides anchorage for the fibers of the periodontal ligament (PDL). The bone approximating the PDL and thus lining the socket walls is called the alveolar bone proper.

Formation of bone is primarily defined as either: endochondral i.e., through ossification of cartilage with mesenchymal cells from blood vessels migrating into the cartilage and thus differentiating into bone-forming osteoblasts; or intramembraneous bone formation, which is when bone develops directly within the mesenchymal soft connective tissue once osteoblasts differentiate and start producing bone matrix. A third bone formation pattern is sutural bone growth which play an important role in the growth of the face and cranium for accommodating the growing organs of the head. Endochondral ossification occurs at the end of bones i.e., long bones, vertebrae and ribs, at the head of the mandible and base of the skull primarily at fetal development and growth in length, but also when bone fractures heal. On the other hand intramembraneous bone formation occurs at multiple sites within each bone during both fetal development and at healing of bony fractures or defects.[63] Importantly the human mandible is by definition formed almost entirely through intramembraneous ossification even if the center of bone formation is the cartilage of the first embryonic arch (a.k.a. Meckel´s cartilage). Similarly in the maxilla the center of ossification is the cartilages of the nasal capsule and the zygomatic process, which have little involvement in the bone formation process. The ossification of the human maxilla is also by definition intramembraneous.[64]

The mandible and the maxilla consist of two separate entities of bone: basal bone and alveolar bone which have different origins. The basal bone of the jaws has no connection to the teeth whereas the portion of the maxilla and mandible that supports the teeth is known as the alveolar process. Importantly it forms when the tooth erupts and has it origin in the dental

follicle.[65] It also gradually resorbs when a tooth is lost. Whether or not this bone can be restored or maintained after a tooth has been lost is issue for scientific dispute and has obvious and important implications with respect to reconstruction of the alveolar bone.

Bone undergoes constant remodeling all through life. The basic multicellular unit (BMU) consisting of osteoclasts and osteoblasts is responsible for the bone remodeling event,[57, 66, 67] which also is the response to trauma such as fractures, healing of bone defects and placement of implants.

Osseointegration

Brånemark coined the term "osseointegration" which describes a condition where a biomaterial (e.g., titanium) is in intimate contact with surrounding vital bone as viewed microscopically (Fig. 1) and bears up with functional loading. It consequently implies that there is no inter-positioned, non-mineralized connective or fibrous tissue between the bone and the implant. Furthermore, successful osseointegration necessitates that the bone surrounding an implant, that carries the load from a prosthesis, does not "react to the presence of the non-biologic component by initiating rejection phenomena."[40]

Brånemark´s original definition of osseointegration reads: "The direct structural and functional connection between ordered living bone and a load carrying implant."[41, 42] Osseointegration is hence mainly a histological definition and can only in part be assessed by clinical and subclinical criteria.[43] Thus one may suspect that an implant is osseointegrated by means of clinical or radiographic evaluation such as immobility and absence of inflammation, but histological evaluation is necessary to verify true osseointegration.

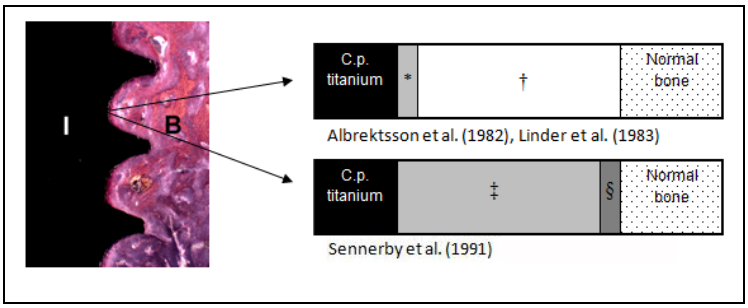

Figure 1. Photomicrograph showing histological evidence for osseointegration (a.k.a. functional ankylosis) of a treaded titanium implant. HTX-Eosin. Light microscope 25X original magnification and on the right hand side schematic illustration of TEM analyses of bone implant interface as cited in the text. *20-40 nm collagen free proteoglycan zone, †100-500 nm randomly arranged collagen filaments with mineral content gradient, ‡100-400 nm amorphous substance zone without collagen or mineral content, § about 50 nm lamina limitans like electron dense line. Schematic illustration in part derived from Albrektsson, Johansson and Sennerby (1994).[44]

Albrektsson et al.[45] examined 33 retrieved titanium implants and reported that implants clinically judged as integrated corresponded to 60% or more of bony contact and 70% or more of bone filling of individual threads.

When analyzing the implant and bone interface at the transmission electron microscopic level (TEM) it has however been suggested that mineralized bone is not in absolute contact with the implant surface. Albrektsson et al.[46] analyzed integration of polycarbonate plugs coated with titanium or gold inserted in the proximal tibial metaphysis of rabbits and harvested at three months. Titanium-coated, as well as the gold-coated, plugs were all surrounded with living bone. The bone appeared to be in "continuum with the titanium interface" whereas a "discontinuous layer of cells separated the gold-coated plugs from the bone." When analyzing the sections with TEM it was however seen that the last 20-40 nm of tissue away from the titanium surface lacked collagen filaments and mainly consisted of partly calcified amorphous ground substance.

Linder et al.[47] also performed an animal experimental study in rabbits to analyze the interface between titanium implants and mineralized bone. Polycarbonate plugs, coated with a layer of pure titanium, were implanted in the tibial metaphysis and left healing for 12 weeks prior to

harvest. The implants were not put in function. At the light microscopic level, implants appeared to be in close contact with bone, but when analyzing sections with TEM at up to 67000X original magnification, a thin (collagen free) gap between the collagen filaments of bone and the titanium surface of approximately 20-50 nm (200-500Å) was seen. The zone stained as normal ground substance containing proteoglycans and with hyaluronic acid and chondroitin sulfate closest to the titanium surface. Using a similar methodology also with polycarbonate plugs, Johansson et al.[48] further explored this zone and showed a significantly wider zone (500-1000 Å) when implants made of titanium alloy were instead used as compared to the zone (200-400Å) when c.p. titanium implants were used. In continuation of these experiments Linder et al.[49, 50] used solid metal c.p. titanium implants inserted in rabbit tibia and reported that a collagen free gap of approximately 50 nm, but also as wide as 1000 nm, was found. Interestingly these authors also included implants of Tivanium® (Ti-6Al-4V alloy, Zimmer Inc., Indiana, USA), Vitallium and stainless steel in the analysis, and it was reported that these metals had similar results with respect to the metal-bone interface as the titanium implants.

Later Sennerby et al.[51] examined ultra thin sections of seven osseointegrated titanium implants that had been in function in humans between one and 16 years. At the light microcopic level, all seven implants were judged to be osseointegrated with mineralized bone tissue in close contact with the titanium surface but at the TEM level an amorphous layer of 100-400 nm in width was seen between the mineralized bone and the titanium surface. It was also reported that in areas with lower mineralization an electron dense zone of about 50 nm was bordering the bone; the authors described this as a “lamina limitans like” line.

It is interesting that the amorphous zone in the human histological samples analyzed by Sennerby et al.[51] was close to ten times wider than what had been reported in previous animal

experiments. One may possibly attribute this either to differences related to type of species (i.e, human vs. animals) or the fact that the implants studied by Sennerby had been in function. One may furthermore argue that the gap was an artifact either from trauma when removing the bloc sections from the surrounding tissues or from shrinkage of tissue and removal of water by the use of alcohol when embedding the histological sections. Though, this argument is probably complicated by the presence of proteglycans in the amorphous zone.

It is repeatedly demonstrated that titanium is biocompatible but the biological properties of titanium are less elucidated on. Except if produced under vacuum, the surface of a titanium dental implant is always covered with an oxide layer with a thickness of at least one to six nano meter. The layer of TiO_2 also increases in thickness with time, and it has been reported that the titanium oxide layer of a dental implant placed in human bone triples from 50 to 150 Å during the first three months after implantation and increases to 2000 Å after about six years in function.[52, 53] However, other authors have presented contradictory findings, showing no increase in thickness over time but instead support a TiO_2 layer, with a constant thickness of five to 10 nm.[54, 55] The crux is that bone and surrounding tissues are not in direct contact with pure titanium but instead with an amorphous layer of TiO_2.[41]

Comments on healing of titanium implanted in bone

The establishment and maintenance of osseointegration represents a dynamic process both with respect to the interaction between the bone walls in the prepared osteoectomy and the newly-installed titanium implant and during the phase of loading with continuous bone remodeling and functional adaptation.[56] Roberts et al.[57] analyzed patterns of bone healing after implantation of spring loaded titanium fixtures in a rabbit animal experimental study. Unloaded implants were compared with implants that were exposed after six, eight or 12 weeks and thus loaded for four, six or eight weeks with stainless steel coil springs with the

aim of comparing bone healing around unloaded and loaded implants. The implants penetrated through the cortical bone into the marrow space. Tetracycline labeling revealed that new woven bone had formed within three days after implantation. During the early weeks of healing, primarily woven unstructured bone was seen. Six weeks after implantation mature lamellar bone with primary osteons had started to replace the woven-bone lattice. From six to 16 weeks remodeling from woven bone to lamellar bone with well-organized and mature secondary osteons were consistently observed. Complete osseous encapsulation was only observed in implants subjected to load. This study consequently suggests that the rate of bone turnover of immature to mature weight-bearing bone (i.e., the remodeling cycle a.k.a. sigma) adjacent to implants in rabbits is about six weeks. Parallel to this the corresponding remodeling cycle in man has been reported to be three to four months.[58]

To address the healing response of c.p. titanium implants inserted in bone, Sennerby, Thomsen and Ericson[59] performed an animal experimental study in rabbits. The implants were penetrating through periosteum and cortical bone, but with the major portion of the implant protruding into the marrow space. Tissue specimens were studied from three to 180 days after implant insertion. After three days very limited bone to metal contact was seen at the cortical section, whereas at the marrow section red blood cells formed a continuous layer along the entire implant surface. After seven days woven bone formation was seen at the part of the implant associated with the endosteal surface of the cortex (i.e., at the junction between the cortical bone and bone marrow). The bone approached the implant, but was never in close contact with the implant; according to the authors, this would indicate that this bone formation did not start at the implant surface. Bone formation at the periosteal surface was first seen after 14 days when remodeling of the old bone with fibrous tissue in contact with the implant surface at the cortical part were also seen. At 28 days the cortical part of the implant was, to a large extent, in contact with dense woven bone. Remodeling was still observed after 42 days.

Remodeling to lamellar bone was completed six weeks to three months after implant insertion which is in agreement with the findings by Roberts et al.[57] At day 90 the bone close to the implant in the cortical regions appeared as mature as the original bone.

From the perspective of osseous formation, bone marrow is an interesting tissue. Bone marrow contains mesenchymal progenitor cells that can differentiate to osteoblasts and is also rich in vasculature with circulating mononuclear precursors that may differentiate into osteoclasts and endothelial cells needed for neo-angiogenesis.[60] In the study by Roberts et al.[57] the part of the implant protruding into the marrow space often had a fibrous capsule. Bone always grew towards the implant surface, hence never it originated from the implant surface. Roberts et al.[57] reported: "a complete endosteal encapsulation of the implants" which was also depicted by figures in the publication whereas Sennerby et al.[59] reported that the section of the implant protruding into the marrow space had a fibrous capsule. These findings are interesting with respect to the origin of the bone in contact with the implant. The bone at the marrow part of the defects may thus either originate from cortex and grow down along the surfaces of the implant into the marrow compartment (appositional bone formation a.k.a. distance osteogenesis[61]) or start to form on the surface of the implant i.e., bone apposition directly onto the implant surface a.k.a. contact osteogenesis.[61, 62]

Both Roberts et al.[57] and Sennerby et al.[59] studied peri-implant bone healing in rabbit tibia using a cortical bone model. One may thus argue that this is not representative of the clinical situation with intraoral implants since implants in these rabbit models were placed in cortical bone; this was in contrast to the combined cortical and cancelous bone morphology representative of the jaw. Berglundh et al.[56] studied bone healing around c.p. titanium implants placed in dog mandibles. These authors also reported presence of newly formed woven bone one week after implants insertion. Importantly, at two weeks the new bone extended only from the cut bone surface ("parent bone") towards the implant surface, whereas

after four weeks, bone formation extended both from the cut bone surface towards the implant surface (appositional bone formation) and also projected directly along the implant surface "remote of the parent bone." The authors suggested that this may be bone formed by contact osteogenesis. This finding is in contrast to the findings by Sennerby et al.,[59] who observed no direct bone formation at the implant surfaces. This discrepancy may though obviously be related to the different animal models used in these studies, i.e., the rabbit tibia defect model involving both the cortical bone and marrow space versus the dog alveolar bone defect model.

Reconstitution of osseous defects

In 1969 Amler[68] studied healing of normal healthy extraction sockets in human histological specimens taken at different time intervals post extraction (Table 1).

Tissue	*Days after extraction*
Clot formation	Same day
Replacement of clot by granulation tissue	7
Replacement of granulation tissue by connective tissue	20
Appearance of osteoid at base of socket	7
Filling of at least two thirds of socket fundus by trabeculae	38
First evidence of epithelization	4
Fusion of epithelium	24 to 35 and more

Table 1. Summary of time sequence of normal human tissue regeneration of alveoli after tooth extraction described by Amler (1969). Copyright © 1969, Elsevier

Seven days after tooth extraction osteoid was seen at the base of the socket which corresponds with the healing pattern described for implant osteoectomies.[56] It may thus be stated that c.p. titanium implants at the least do not obstruct normal bone reformation in humans. Whether or not titanium improves osseous healing by e.g., osteoconduction is still unclear and an issue for discussion.

The mechanisms of bone healing are quite similar to embryonic osteogenesis and growth, and since bone has a unique spontaneous healing pattern the optimal regenerative methodology would be to harness the internal regenerative capacity of bone.[69] So far it has not been possible to develop strategies that completely mimic these complex biological events, and bone reconstructive strategies have for the most part instead been directed towards substituting bone with various types of biomaterials. Over the years a vast number of osseous reconstructive strategies have been suggested.

Autogenous and allogeneic bone

The most obvious strategy and still the gold standard for osseous defect reconstruction, is using a graft constituting of bone harvested from a donor site within the same patient, a.k.a. autogenous bone graft. Within the field of periodontology the use of an autogenous bone graft was first described by Hegedüs in 1923,[70] who used bone blocks from the tibia to reconstruct bone lost due to "advanced pyorrhea" (i.e., advanced periodontitis). Hegedüs reported some evidence for increase in alveolar bone height as well as decrease in mobility. In 1964 Hirakawa and Uji[71] presented the first cases with autogenous bone harvested intraoraly and used for grafting of defects caused by cysts, root resection, filling of sockets after extraction and mandibular fractures. In 1965 Nabers and O´Leary[72] presented a classic paper detailing the successful use of autogenous particulate bone for grafting periodontal osseous defects. The graft material was harvested intraorally, from the same surgical area as where the recipient defects were localized. The limitation of using autogenous bone lies in the restricted bone volume at intraoral donor sites, which sometimes may not be sufficient for complete fill of the recipient site. Harvesting donor bone also involves an extent of patient morbidity, in that quite often a second surgical site is needed. Adverse events such as nerve damage,[73] mucosal defects at the donor site or damage to neighboring structures, such as apices of teeth,

are also risks involved in the harvesting of autogenous bone. Using extraoral donor sites such as iliac bone is extraordinarily involving and has also been reported to be associated with an increased risk of root resorption.[74] The utilization of bone transplants harvested from another individual of the same species (homogenous bone, allogenic bone) gained a lot of momentum during the 1970s. In 1958 Green[75] published a case series of six patients with intraoral defects reconstructed with particulate grafts, either autogenous bone or fresh frozen homogenous bone and were optimistic regarding the clinical and radiographic results. In 1970 Hiatt and Schallhorn[76] published a study on usage of human bone allografts for treating periodontal defects. In contrast to previous attempts with allogenic bone grafting, these authors had blood group- and human lymphocyte antigen (HLA) typed the patients and thus cross-matched the donor and the recipient patients. Since immune rejection is an obvious problem when using allogenic bone grafts, developing methods for altering the antigenicity of allogenic bone became an important focus in science. An animal experimental study in dogs by Kreuz et al.[77] evaluated grafting of osseous defects with freeze-dried allogenic bone. The freeze-dried bone grafts were reported to incorporate well with surrounding bone tissue. Due to the freeze-drying process, it was possible to make allogenic bone storable in room temperature in bone-tissue banks. In an animal experimental study in rabbits and later in a human study, Friedlander, Strong and Sell[78, 79] furthermore demonstrated that the antigenicity of bone was markedly reduced by the freeze drying process, which had been suggested to distort the three-dimensional presentation of HLA.[80]

Freeze-dried bone allografts (FDBA) are considered to be osteoconductive.[80] It has also been suggested that hydrochloric acid demineralization of FDBA (i.e., to DFDBA) would project factors stimulating bone growth, such as bone morphogentic proteins, and thus make the allograft osteoinductive.[81-83] This has also been demonstrated by means of histology,[84, 85] albeit opposing results have later been presented.[86] The presence of bone morphogenetic

proteins (BMP)[87] and the osteoinductive potential of DFDBA has been debated and at the least seem to be dependent on factors such as preparation[88] and donor age.[89] The major concern, although for the most part considered solely as a hypothetical risk,[90, 91] is disease transmission. For such reasons, developing and testing non-human bone substitutes have gained a lot of focus throughout the years.

Xenogenic bone

Biological tissue transferred between different species is known as xenogenic. Since bone structure is similar in most mammals, it was an early focus towards experimenting with the use of xenogenic bone for reconstitution of bone defects in humans. Already in 1934 Beube and Silvers[92] had performed an animal experimental study in dogs where surgically-created defects were filled with boiled cow bone and a more rapid healing in the cow bone filled defects was reported.[93, 94] In a subsequent series of case reports, the successful reparative treatment of osseous defects in humans with boiled cow bone was presented.[94] A number of later investigations developed and examined techniques other than boiling for extracting the organic matrix of cow bone, such as ethylene diamine and alcohol,[95 96] with the aim to solely implant the inorganic component of the xenogenic bone and thus avoid provoking an immunologic reaction. In an animal experimental study in rhesus monkeys, Losee and Boyne[95] reported that ethylene diamine-treated inorganic cow bone grafts were well accepted by the host bone. Melcher[97] later presented contradictory findings from a clinical study showing that even if the implanted cow bone was well accepted by the host tissues, much of the material persisted non-resorbed, and sequestration of the material was seen for more than three years after implantation. It was also reported, quote, that: “the presence of the material tends to hinder rather than assist the healing of bone.” Later methods of deproteinizing bovine bone suggest more positive results. Deproteinized bovine bone mineral (DBBM) is as of today,

a commonly-used bone substitute and the osteoconductive properties and potential as a bone replacement graft have been demonstrated repeatedly[98-100] whereas other authors present more negative findings.[101, 102] A second example of a xenograft is hydrothermally and chemically derived HAp from the exoskeleton of sea coral. By architecture the exoskeleton of corals resembles natural bone and thus potentially could be used to mimic natural bone structure and work as an osteoconductive bone substitute. Positive outcomes have been demonstrated both in animal experimental[103] as well as in clinical studies.[104-107]

Synthetic bone substitutes

A synthetically derived material is available in ample amounts without having to perform a harvest procedure as compared to autogenous bone. Furthermore there is no disease transmission risks with a synthetically derived biomaterial as compared to the potential concerns with allografts and xenografts.[109] Synthetically derived bone substitutes includes alloplasts, such as a wide range of ceramics but also polymers and metals.

Alloplastic bone substitutes

Alloplasts are inorganic synthetically derived bone substitutes fabricated with the aim to mimic the chemical and/or the morphological composition of the mineral phase of bone for usage as an osteoconductive bone replacement biomaterial.[108] Alloplasts may be used either alone as a bone void filler or as an extender of an autogenous bone graft.[108] A range of alloplasts have been presented throughout the years. Examples of alloplasts are ceramics such as calcium sulfate ($CaSO_4$) a.k.a. "gypsum"),[110] calcium phosphate ($Ca_{10}(PO_4)_6OH_2$) e.g., triple calcium phosphate as presented in an animal experimental study by Albee and Morrison[111], synthetically derived HAp and tricalcium phosphate.[108] Other examples of

ceramic alloplasts are bioglasses (i.e., SiO_2, Na_2O, CaO and P_2O_5), and polymers such as polymethyl-methacrylate and hydroxy-methylmethacrylate.[112]

Titanium from the perspective of osseous defect reconstitution

It is interesting to speculate on the biological properties of titanium from the perspective of osseous reconstruction. Little is known about the biological performance of a titanium body when implanted into vital living tissue with the aim to reconstruct bone.

In studies on bone and blood cell reactions to titanium, it has been shown that the surface structure and surface properties of an implant, such as oxide thickness, chemical composition and roughness, has an impact on bone response to an implant surface,[113-122] thus affecting the release of growth factors and cytokines that are of importance for bone healing.[60, 123]

A study by Hong and co-workers[124] used an *in vitro* whole blood chamber model to study platelet adhesion and platelet activation of titanium and titanium-nitride with various surface roughness as compared with PVC and steel. Only the titanium surfaces induced clotting by activation of the complement system as seen by the generation of thrombin-anti-thrombin (TAT). When analyzing effects of surface roughness it was shown that the rougher surfaces induced more generation of TAT than the smooth surfaces. Moreover it was also shown that blood contact with titanium lead to a more pronounced surface binding of platelets and increased platelet activation, as reflected by higher levels of β-thromboglobulin and platelet derived growth factor (PDGF) as compared to the other tested materials.[124] It has previously been shown that PDGF and other platelet α-granule proteins such as transforming growth factor-β (TGF-β), are substantial promotors of bone growth.[125] [126] Based on these observations it was suggested by these investigators that the osteointegrating properties of titanium may to the least in part be the result of the potent activation of the coagulation system. One may thus speculate that increasing the total titanium surface exposed to blood,

e.g., by using porous particles may further enhance platelet activation. Adapting these observations to the field of regenerative surgery, one may consider using a strategy with a bone substitute made of titanium with a surface as large as possible to promote new bone formation in osseous defects. Porous titanium granules (PTG, Natix®, Tigran Technologies AB, Malmö, Sweden) represent one such titanium biomaterial which may potentially have properties corresponding with the above discussion (Fig. 2).

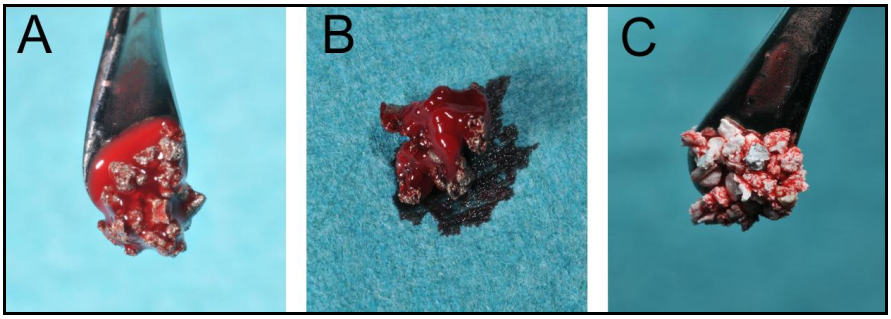

Figure 2. A) PTG and blood. B) PTG and blood. Note the blood clotting. C) Heat oxidized PTG (WPTG) and blood.

A porous titanium granule is 700-1000 μm in diameter but the total titanium surface of one granule is approximately two cm^2, which accordingly leads to a significant area for blood-to-titanium contact.

There has been anecdotal evidence supporting the theory that PTG can integrate in human bone and potentially induce osseous healing. However, the scientific rationale for utilization of porous titanium granules for reconstructive treatment of osseous defects has been scarce and limited to case reports[127 128] as well as one animal experimental study.[129] The material was first used by orthopedic surgeons in conjunction with fixation of titanium hip stem prostheses.

Alffram et al.[128] reported on findings from a series of five cases with titanium hip stem prostheses fixated with PTG instead of regular acrylic cement. Both clinical and radiographic

analyses revealed stable prostheses after nine to 15 years follow-up. One post mortem autopsy was also retrieved. The histological analysis from this patient as well as computerized tomography of the treated area from three patients supported the clinical and radiographic findings and revealed that PTG became incorporated in bone (Fig. 3).

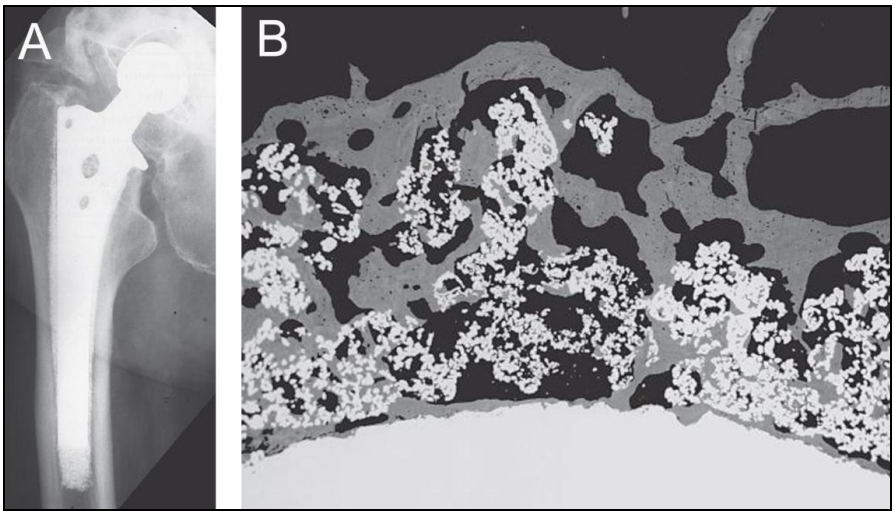

Figure 3. Derived from Alffram et al.[128] A) 10 year follow-up radiograph of a subject with a hip stem implanted in a bed of PTG B) Backscattered electron micrograph showing the interface between the titanium hip stem, PTG and the surrounding trabecular bone. Copyright © 2007 TAYLOR & FRANCIS

Turner et al.[129] performed an animal experimental study in 10 dogs with the aim to perform a histological evaluation of PTG when utilized for cementless fixation of a hip replacement femoral stem. At termination, six-months after surgery, nine out of ten dogs had healed uneventfully and the histological analysis showed PTG incorporated in bone. In a clinical pilot study, Jónsson and Mjöberg[130] used PTG with the aim to achieve a non-resorbable reduction of depression fractures of the lateral tibial plateau. Four cases were followed for up to six months, and it was reported that the radiographic results indicated no loss of plateau height (Fig. 4) after treatment. The clinical stability was also reported to be excellent. For the orthopedic applications the size of the granules has been one to two millimeters.

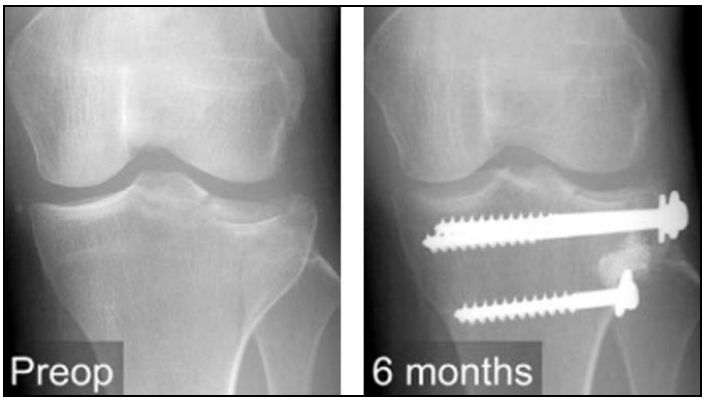

Figure 4. Preoperative radiograph and six months control radiograph after treatment with PTG of a depression fracture of the lateral tibial plateau From Jónsson and Mjöberg[130] Copyright © Upsala Medical Society.

PTG of the size 0.7 to one millimeter have been used within the field of oral and maxillofacial surgery. In a case report by Bystedt and Rasmusson,[127] results after follow-up of Tatum type sinuslifts[131] with PTG performed in 16 patients were presented (Fig. 5). In total 23 implants were installed. Four of the patients had their implants installed in a second surgical procedure. After a follow-up period of 12 to 36 months, three implants were lost which gives a survival rate at the implant level of 87%. Noteworthy were two failures in the two-stage group which consequently may be important from the perspective of preparation of an osteotomy by drilling into PTG potentially incorporated in bone. It is important to further elucidate on this, prior to clinical usage of the material for augmenting or preserving implant sites. Holmberg et

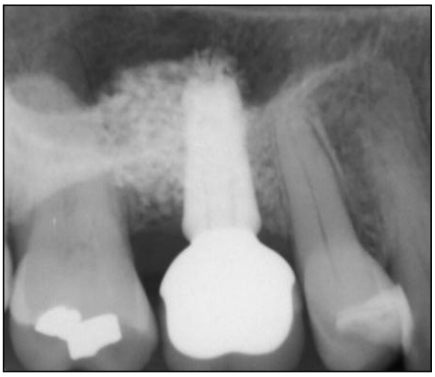

Figure 5. Tatum type sinuslift performed with PTG. Five years control radiograph. Compliments of Dr. Hans Bystedt.

al.[132] reported on one case where PTG were used in conjunction with a split crest technique for horizontal expansion of a severely resorbed maxillary dento-alveolar ridge. At the 12 year radiographic follow-up, an average of less than two mm marginal bone loss was found and the implants were stable. Sabetrasekh et al.[133] performed an in vitro analysis of the biological and morphological properties of PTG versus three other bone replacement biomaterials. It was shown that PTG performed significantly better with respect to cell proliferation rate compared to DBBM. When characterizing the architecture of the PTG particles it was also shown that the majority of the pores were larger than 100 μm and with a mean pore size of 241.6 μm as compared with 129.9 μm for DBBM. Pore size seems to be important from the perspective of bone growing in to the bone substitute particles and a study by Klawitter and Hulbert shows that a size of interconnecting pores above 100 μm is required for mineralized bone growth into a biomaterial whereas osteoid was demonstrated in pore sizes down to 40 μm.[134] The biomaterial used in this study was a ceramic made of calcium aluminate. Later studies, instead testing outcome of pore size in perforated titanium plates with pores down to the size of 50 μm, have failed to verify the above threshold values for mineralized bone ingrowth.[135] One may thus speculate that the in-growth of mineralized bone also may depend on the type of biomaterial used.

By convention a bone substitute should resorb and be replaced by new bone. It is however interesting to reflect on the potential advantages of using a non-resorbable bone substitute. Since osseous growth and maturation is a long-term process it may well be that a biomaterial acting as a permanent growth substrate may be an advantage as compared with resorbable biomaterials. It is noteworthy that the scientific data on resorption time and resorption pattern of bone substitutes are conflicting. Hallman, Lundgren and Sennerby[100] compared human histological results from biopsies taken six months and three years after a sinus augmentation with DBBM (Bio-Oss®, Geistlich Pharmaceutical, Wollhausen, Switzerland) mixed with 20%

autogenous bone and demonstrated no resorption of the DBBM particles neither after six months nor after three years. Interestingly the new bone to DBBM surface contact had increased from 28.8% at six months to 54.5% at the three year biopsies. When analyzing the biopsies taken after six months, the bone was predominantly immature and woven, whereas it was mainly mature and lamellar at three years. In a sequel to the Hallman, Lundgren and Sennerby study, Mordenfeld et al.[136] retrieved biopsies from the same patients, 11.5 years after the augmentative surgical procedures and reported 17.3% remaining non-resorbed DBBM particles. Furthermore, no significant differences with respect to surface length and area of the DBBM particles were seen at the 11.5 years biopsies, as compared with the biopsies retrieved after six months and after three years, or as compared to virgin particles. Similarly, Iezzi et al.[137] reported that remaining anorganic bovine bone (Osteograf, Ceramed, Lakewood, USA) particles were demonstrated in a biopsy from a patient who had been treated with a sinus lift augmentation 14 years earlier.

Intraoral osseous defects being potential candidates for reconstructive therapy

Within the field of dento-alveolar surgery a number of osseous defect types are potential candidates for reconstructive therapy. The bone of the alveolar process is formed by the development and the eruption of teeth and later dependent on teeth. The consequence of tooth loss is accordingly and by principle physiological loss of alveolar bone. With regard to pathological intraoral bone loss, periodontal disease is the most common cause. Advanced periodontitis affects approximately 10-15% of the population.[138-140] A number of cross-sectional studies report that the relative contribution of advanced periodontitis as the reason for extractions of teeth is between 18-36%.[141-146] Osseous defects as a consequence of tooth loss may be detrimental to the individual patient, for example with respect to the plausibility of replacing lost teeth with dental implants.

Peri-implantitis and peri-implant osseous defects

Notwithstanding the high success rate reported for dental implants[147-149] evidently a relatively high number of implants develop loss of attachment over time. Peri-implantitis has been defined as bone loss of dental implants induced by infection and was described by Mombelli in 1987.[150] Throughout the years a numbers of diagnostic criteria for this disease entity have been suggested. It is probably important to distinguish between early bone loss, which may be due to osseous remodeling after implant placement, and which takes place early after implant seating. This early bone loss, which usually has been described to take place within the first year after seating of the implants, has been considered to be physiologic in nature. Albrektsson et al.[43] stated that one criteria for dental implant success, should be less than 0.2 mm annual vertical bone loss, following the implant´s first year of service. With respect to loss of peri-implant attachment, this success-criterion implies that: (1) implants may or may not lose bone the first year of function due to physiological conditions not possible to withstand, (2) that 0.2 mm of yearly bone loss is within physiological limits and should be considered acceptable. A yearly bone loss of 0.2 mm over e.g., 25 years would by such criteria connote five mm of lost peri-implant attachment and potentially an additional one mm under the circumstance that one mm of bone also was lost during the first year of function. In this context it is interesting to relate to animal experimental studies on ligature-induced peri-implantitis showing that after a lesion has been established it continues to progress in most cases (i.e., after ligature removal)[151] even if it has been suggested that the extent of the continued bone loss varies depending on the implant surface type.[152-154] Whether such findings also relates to the clinical situation, have not been completely examined and further studies on this theme will be of major importance, since this may relate to the necessity to intervene against established peri-implant lesions.

The definition of peri-implantitis has changed throughout the years. Lately Zitzmann and Berglundh[155] proposed that the joint term for inflammatory reactions in the tissues surrounding an implant should be peri-implant diseases, while peri-implant mucositis was defined as quote: "inflammation in the mucosa at an implant with no signs of loss of supporting bone." Peri-implantitis was defined as inflammation in the mucosa with loss of supporting bone.

It is also important to distinguish between peri-implantitis, which describes the disease entity according to the above definitions, while a "peri-implant osseous defect" is the bony defect potentially in need for reconstructive therapy after finished causative therapy. The osseous defect, rather than peri-implantitis, as a disease entity is reconstructed, while the disease per se is treated causatively. It is important though to know that so far no non-surgical treatment method has been shown to be effective in hindering defect progression, and the lesions caused by peri-implantitis are usually in need of surgical intervention for achieving a sufficient result. So far there is scarce evidence based research data to endorse a specific treatment strategy for peri-implantitis. The dental scientific community consequently needs to evolve within this field (Appendix II).

The concept of re-osseointegration

The ultimate outcome when treating peri-implant bone loss is re-osseointegration. Renvert et al.[156] defined re-osseointegration as: "formation of new bone onto a previously biofilm-contaminated implant surface." This definition implies that histology, or plausibly other high resolution methods for analyzing the bone to implant interface, is needed when evaluating if a specific treatment regime promotes re-osseointegration. Several animal experimental studies suggest that it is possible to accomplish this treatment goal after various treatments of

experimentally induced peri-implant osseous defects[157-166] but there has been no human histological evidence for re-osseointegration presented in the literature.

Periodontal furcation defects

Classification of furcation involvement is based on the degree of horizontal loss of osseous support in the inter-radicular area, with degree I being up to one third of the width of the tooth, degree II more than one third but not encompassing the total width of the tooth and degree III being a through-and-through destruction of the periodontal support.[167]

A great number of studies have investigated different treatment protocols for reconstructing lost periodontal support in furcation defects (Appendix I and III). Clinical studies by Schroer et al.[168] and Kalkwarf et al.[169] compared closed versus open flap debridement (OFD) and showed no beneficial effects of non regenerative surgical therapy as compared to scaling and root planning alone. Noteworthy, the study by Kalkwarf et al.[169] demonstrated that neither non-surgical nor non-regenerative surgical techniques were able to hinder progression of horizontal bone loss as demonstrated after two years of periodontal supportive therapy.

Guided tissue regeneration (GTR) is the regenerative approach for degree II furcation defects with most scientific documentation. A systematic review by Murphy and Gunsolley[170] report that GTR procedures for regenerative treatment of degree II furcation defects resulted in a greater gain in clinical attachment level (CAL), as well as a greater reduction in pocket depth (PD) as compared to OFD alone. The included studies had a follow-up of six months or more. The mean gain in CAL for the studies finally included was 1.9 mm with 3.1 mm of reduction in PD.

General hypothesis of the thesis

The general null hypothesis (H_0) of the thesis was that: Reconstitution of osseous defects with porous titanium granules will not significantly improve the defect resolution as compared to control defects left empty. The alternate hypothesis (H_A) of the thesis was that: Reconstitution of osseous defects with porous titanium granules will significantly improve the defect resolution as compared to control defects left empty.

General objective of the thesis

The general objective of the thesis was to investigate the potential of PTG when used as a bone substitute material in peri-implant and periodontal osseous defects.

Specific aims of the thesis

The specific aims of the studies included in this thesis were:

A) To compare the osteoconductive properties of PTG with controls left empty in osseous defects adjacent to c.p. titanium implant surfaces. (*Paper I*)

B) To analyze the biological performance (bone formation, resorption and inflammation) of PTG in osseous defects adjacent to c.p. titanium implant surfaces. (*Paper I*)

C) To compare the potential of PTG with sham and DBBM in the reconstructive treatment of surgically created buccal, degree II furcation defects. (*Paper II*)

D) To evaluate periodontal ligament regeneration and the presence of root resorption lacunae, when PTG is placed in surgically created, buccal, degree II furcation defects. (*Paper II*)

E) To analyze the osseous reconstitutive properties and potential to support re-osseointegration of PTG when used as a bone substitute in the reconstitution of a human peri-implant osseous defects. (*Paper III*)

F) To compare clinical and radiographic results from PTG implanted peri-implant osseous defects with non-implanted controls. (*Paper IV*)

G) To evaluate clinical and radiographic performance of PTG when used as a bone substitute in the treatment of mandibular degree II furcation defects. (*Paper V*)

Materials and methods

This thesis consists of a series of studies analyzing the performance of PTG using animal experimental and clinical research models. In paper I, calibrated osseous defects were prepared in the tibias of 24 New Zealand rabbits, which were grafted with either metallic or oxidized porous titanium granules (PTG or WPTG respectively), whereas control defects were left empty (sham). The defects were closed with a submerged coin shaped titanium implant and left for healing for four weeks. After healing, the implants were removed and the tissue formed onto the implant surface was analyzed using RT-PCR. Wound fluid was sampled at time of harvest with 6.25 mm circular filter papers. Samples were analyzed at the same day by spectrophotometry. Histological and micro-computed tomography (microCT) analyses were also conducted to study osseointegration of titanium granules, as well as osseous reformation in defects.

In paper II buccal degree II furcation defects were surgically created in maxillary premolar teeth in adult, female, mini pigs and filled with PTG or DBBM, or left empty (sham) (Fig. 6). After six weeks of healing, pigs were euthanized. Teeth with defects were excised en bloc and analyzed by microCT and histology.

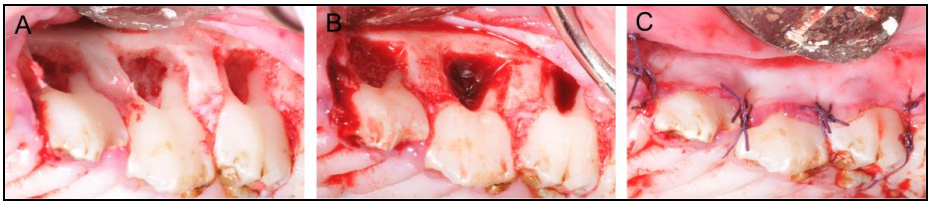

Figure 6. Furcation defect model used in paper II. **A)** A muco-periosteal flap was raised and buccal degree II furcation defects were surgically created in premolar teeth P2, P3 and P4, The periodontal ligament and root cementum were removed using curettes and surgical diamonds. Notches were made at the most apical part of the defects. **B)** Defects were randomized to either grafting with DBBM, PTG or left empty (sham). **C)** The surgical sites were closed with Vicryl® 4.0 resorbable sutures.

Paper III is a human histological analysis of an implant that 12 months earlier underwent PTG reconstructive treatment of a peri-implant osseous defect. Analyses of the biopsy were performed by means of microCT and histology. Presence of phosphorous and calcium in the newly formed bone were validated by scanning electron microscopy using an energy dispersive x-ray analyzer (EDAX).

Paper IV is a prospective, randomized, case-control, clinical study of 12-months duration (Fig. 7) comparing open-flap debridement and surface decontamination with titanium curettes and 24% EDTA gel or additional insertion of PTG. Implants were submerged and allowed to heal for six months. Probing pocket depth (PPD), bleeding on probing, implant stability using resonance frequency analysis (RFA), and radiographic evaluation were performed at baseline and at 12 months.

In paper V surgical intervention with PTG used as a bone graft substitute was performed in 10 patients with 10 mandibular degree II buccal furcation defects. Clinical parameters (probing depth (PD), CAL, gingival recession (GR), gingival index (GI), bleeding on probing (BoP), horizontal and vertical bone sounding) and radiographic measurements of vertical furcation height were compared between baseline (pre-surgery) and six and 12 months (post-surgery).

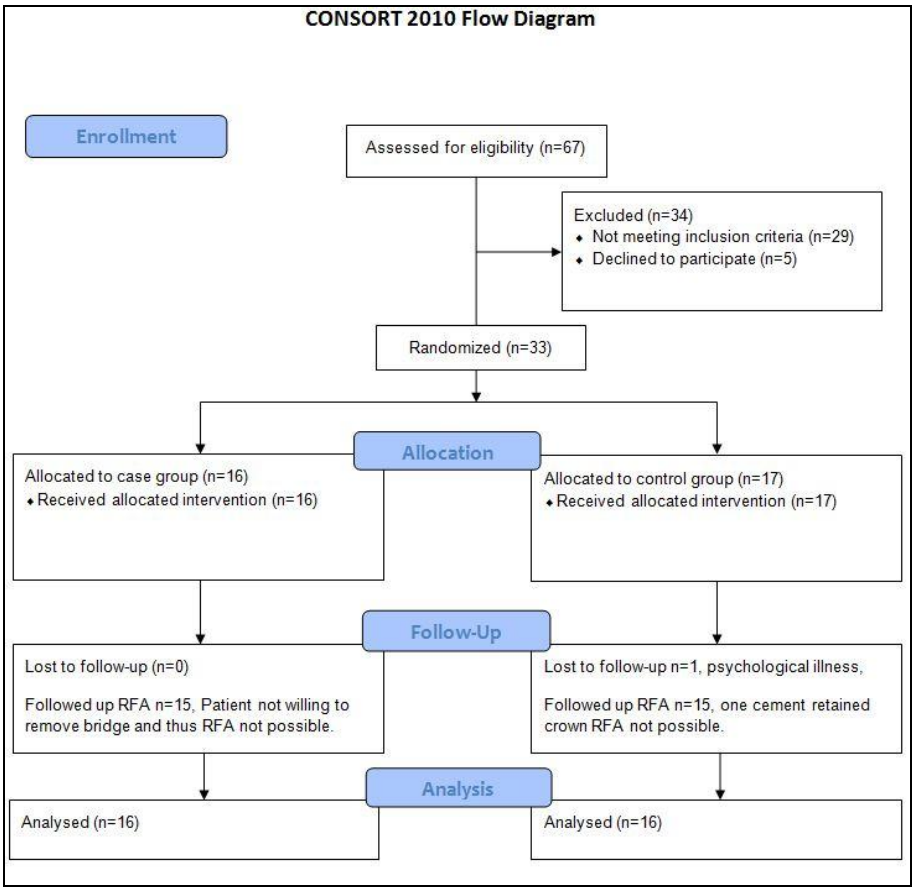

Figure 7. Consolidated Standards of Reporting Trials (CONSORT)[171, 172] flow diagram outlining the study presented in paper IV. Flow diagram format: Copyright © 2010, British Medical Journal Publishing Group

Summary of results

Paper I

Significantly more new bone formed in PTG and WPTG treated defects compared to sham. The new bone grew both through the porosity of the granules and onto the implant surface. The WPTG group showed significantly less expression of key inflammation markers, but with no significant difference in a marker for necrosis. The WPTG group also showed a significant increase in collagen-I mRNA expression as compared to PTG.

Paper II

The histological analysis showed significantly more vertical bone formation in both PTG and sham groups compared with DBBM treated defects (P <0.01). MicroCT analysis showed significantly more bucco-palatal bone formation in furcations implanted with PTG compared with the DBBM and sham (P <0.05). Bucco-palatal cylindrical microCT cores demonstrated a mean defect fill of 95.2% for PTG-implanted defects, which was significantly greater than sham (62.3%) and DBBM (66.5%) (P <0.001) treatments. Significantly more regenerated PDL was seen for sham than DBBM treated defects (P <0.05) (Fig. 8).

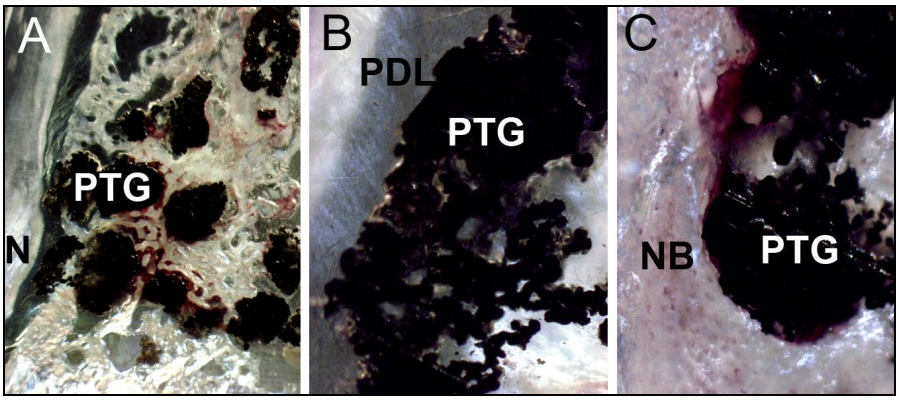

Figure 8. Photomicrograph of histological sections from a furcation treated with PTG in study II **A)** Polarized light 25X. **B)** Polarized light 100X. **C)** L.M. 100X. N =Notch, NB =New Bone, PDL =periodontal ligament fibers, PTG =porous titanium granules.

Root resorption lacunae were small and infrequent and did not differ between groups.

Paper III

No signs of objective side-effects such as exfoliation of graft material or overt inflammatory reaction were seen at any time-point during the follow-up. No subjective side-effects such as pain or discomfort exceeding similar type surgical procedures were reported. The implant was partly covered with mucosa at time of excising the biopsy and PPD measurements could thus not be performed. Only very minor signs of mucositis, and no signs of suppuration of pus, were observed at the time of implant removal.

The baseline radiographic mesial and distal defect heights were 9.24 mm and 5.70 mm respectively, while the corresponding radiographic defect heights at the 12 months terminal evaluation were 0.97 and 0.74 mm respectively. Horizontal microCT sections demonstrated PTG embedded in new bone and areas of re-osseointegration of the implant, i.e., new bone formed between the granules and the implant, and new bone in contact with the treated implant surface (Fig. 9).

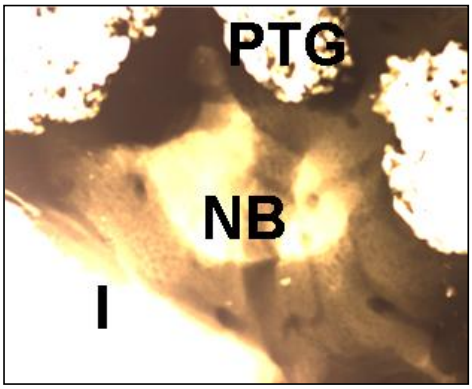

Figure 9. Human biopsy from patient reported in paper III. Microradiograph showing bone in close contact with PTG particles, bone between PTG and the dental implant surface and in close contact with the treated implant. L.M. 25X

The SEM analysis demonstrated areas where the implant was re-osseointegrated with new bone growing onto the implant surface, onto the PTG and into the porosities of the granules. The SEM-EDAX element analysis demonstrated calcium and phosphorus in the tissue embedding the PTG and implant giving further support for presence of calcified tissue in the spaces between the PTG and between PTG

and the dental implant.

The histological analysis demonstrated PTG well integrated in zones of woven and lamellar bone. Clear presence of lamellar bone between the granules and the implant surface and areas of contact between the new bone and the implant surface were furthermore demonstrated i.e., re-osseointegration. Instances of narrow zones of fibrous tissue were however also observed between the new bone and the implant surface.

Paper IV

Change in radiographic defect height and percent fill of the peri-implant osseous defect significantly favored patients treated with PTG (P <0.001). Both treatment modalities demonstrated significant improvements in PPD (P <0.001), but no significant differences between groups were observed. The PTG treated implants showed an increase in RFA of 1.6 implant stability quotient (ISQ) units compared with a decrease of 0.7 ISQ for the control group (n.s.). No adverse effects were associated with PTG treatment.

Paper V

With respect to vertical and horizontal bone sounding measurements, CAL and GR, no significant improvements between baseline and the 12 month examination were seen. Both PD and radiographic vertical furcation height were significantly reduced between baseline and 12 months. When comparing the baseline and 12 months data, a significantly lower GI score was seen but the BoP score was unchanged for the same time interval.

None of the treated teeth showed radiographic signs of root resorption.

Discussion

Methodological considerations

Research models

Two different animal experimental models and two clinical models were used to elucidate on the specific aims of this thesis. In paper I, a New Zealand White rabbit (Oryctolagus cuniculus) tibia defect model were used whereas a mini pig (Sus Scrofa) furcation defect model where used in paper II. Animal models are primarily hypothesis generating and clinical studies are always necessary to further explore or verify the experimental findings in humans. In paper III and IV the use of PTG as a bone substitute in the surgical treatment of peri-implant osseous defects was investigated. In paper V, the utilization of PTG as a reconstructive biomaterial for periodontal furcation defects in mandibular molars, was investigated.

The animal model in paper I was only performed to get an initial idea of the performance of the novel biomaterial prior to the execution of preclinical investigations (paper II) and clinical testing (paper III-IV). In paper I the material was tested in a closed implant defect model without any load from neither teeth, nor implants, whereas the dental furcation defect model in study II both involved load from occlusion forces as well as potential inflict on the healing from the oral environment.

One of the major advantages with performing animal experimental studies is standardization of the experimental system. The choice of animal model is obviously related to the objective of the study. When aiming at evaluating **osteoconductivity and biocompatibility** of a material, bone defects large enough to avoid spontaneous healing (a.k.a. critical size defect[173]) and small enough that also minor relative improvements can be detected.[174] Two methods exist for creating critical size defects:

(1) To create a defect and thus prevent healing by for example insertion of a foreign body such as a ligature or dental impression material.

(2) To create a defect large enough that spontaneous healing does not occur.

Only the second method creates a true critical size defect and is thus often considered to be the best method of choice since the first method involves too many uncontrollable variables (e.g., infection, nutritional and metabolic abnormalities) while the second model optimizes the bodies physiologic response.[173] The less the number of uncontrollable variables involved in a model the easier it is to create calibrated defects. This saves number of animals involved in the study but also makes the testing system more rigid. The uniform, radial geometry of the cylindrical defects used in paper I, makes them highly defined. By using a closed system it is also possible to avoid variation in external encroachment affecting the healing pattern. In paper I, defects three millimeters in diameter and five millimeters in height were created in the tibia of rabbits. Two defects were created in each leg. The defects were covered with a titanium disc to study healing in close proximity with a titanium implant surface (Fig. 10). The variation between defects is minimal. The rabbits may plausibly have been somewhat young since they were only eight months, while rabbits has been reported to be skeletally mature at 10-11 months,[174] albeit other authors have reported that female New Zealand white rabbit is skeletally mature already after six to seven months.[175, 176] It is in this context important to note that all animals were of the same age which probably canceled out for this hypothetical consideration. In paper I an animal experimental model was used to assess the performance of PTG in proximity to a titanium surface and for analyzing bone growth in PTG treated defects. The major crux with this study was to assess osteoconductivity and biocompatibility of PTG and the findings from this closed defect model in rabbit tibia bone should thus not be directly derived to the related intra oral clinical situation with a dental implant with an infected osseous defect communicating with the oral cavity.

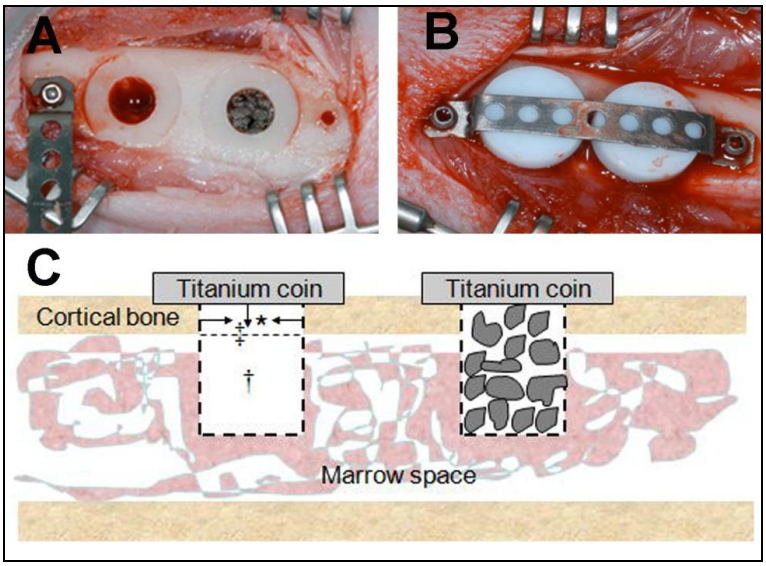

Figure 10. Tibia defect model used in study I: **A)** Intra-surgical pictures of test-device (PTG) versus control defects left empty. **B)** Defects are covered with coin-shaped titanium implants, covered by a teflon cap and stabilized by a pre-shaped titanium plate fixed by titanium screws. **C)** Indexes used for the histomorphometrical analyses *Index A. Horizontal dimension of regenerated peri-implant cortical bone. †Index B. New bone in the marrow space compartment. ‡Index C. Vertical dimension of new peri-implant cortical bone. Image in part derived from Wohlfahrt et al. 2010 with Copyright © 2010 John Wiley & Sons Inc

Rabbits have a much faster bone healing rate than humans,[177] and the bone forming rate has been reported to be approximately three times as fast in rabbits as in humans.[57] Roberts et al. report that the BMU in rabbits femur is 1300 μm and moves at a speed of 220 μm per weeks time and hence a total bone forming period (a.k.a. sigma[66]) of six weeks. The average BMU in human cortical femur bone has been demonstrated to be 3700 μm.[178] Sigma in humans (rib bone) is four to five months.[57] One may still argue that the study was short in length. Again it is important to note that the major task was to compare three different treatment modalities to get a basis for testing in more advanced animal experimental models which to a greater extent mimic the clinical situation (paper II).

In paper II the aim was to perform a preclinical test of PTG as a reconstructive material and a mini pig (Sus Scrofa) furcation defect model was used. One of the secondary objectives was

to perform a safety assessment and test if PTG in close narrowness to root surfaces may cause root resorptions. A challenge was to distinguish between root damage caused by excessive preparation of the root surfaces in conjunction with creating the furcation defects and true resorption lacunae. It is important to stress that the model used in paper II only in part mimic the clinical situation of furcation defects caused by periodontal disease. The furcation defects created were significantly larger than what is typically seen in humans. The aim with this was to extend the defects to critical size.[173] Moreover the model used does not involve infection with representative periodontal pathogens nor does it comprise a state of chronic inflammation. When performing analyses of different treatment modalities in an animal experimental setting, it is of importance to use a model with a high sensitivity and by this: (1) limiting the number of animals but also reaching sufficient statistical power and (2) limiting the study time to keep away from unnecessary morbidity for the animals. Under such circumstances, calibrated defects, homogenous in defect size will be important.[179] It has though previously been argued that neither furcation defects which are caused by ligature induced infection, nor naturally occurring furcations will be sufficiently homogenous for optimal comparison between test and control groups.[180, 181] Creating surgical defects and performing experimental treatment in the same surgical session is also less stressing for the animals than other models with experimentally induced defects that has previously been described in the literature (Appendix I).

In paper III the performance of PTG when used for reconstruction of a peri-implant osseous defect was evaluated by means of histology. The major crux was to explore if a dental implant affected by peri-implantitis can re-osseointegrate. By definition osseointegration implies that the implant is in function. One may thus argue that since the implant was never put in function after the defect had been reconstructed with PTG the complete definition of re-osseointegration has not been fulfilled. It is though argued that the definition re-

osseointegration has previously been used for animal experimental studies exploring the principle of re-osseointegration and in these studies the implants were never put in function after treatment and prior to harvest.[166, 182, 183]

Few randomized clinical trials have been performed on the treatment of peri-implantitis and on reconstructive surgery of peri-implant defects (Appendix I). One concern with clinical studies on peri-implant defects is to ascertain balance between test and control groups. The reason for this is multi-plethoral (for a comprehensive review see Esposito et al. 1998.[184]):

(1) It is often difficult to securely determine the etiology of a peri-implant defect. To do so it will be necessary to compare radiographs taken at the one year control with radiographs taken at time of screening for the study. By experience such radiographs are not often attainable. So called "physiological" loss of bone due to bone remodeling during the first year of function is not the same entity as bone loss caused by peri-implantitis and may not even need treatment if no progression is demonstrated over time.

(2) The author´s experience is that the peri-implant defect morphology varies to a much greater extent than what is generally understood. The optimal situation would certainly be a split mouth clinical study. Out of 66 potential subjects screened for study IV only one had a matched pair of peri-implant osseous defects.

(3) Technical contributing factors must be completely elucidated on and potentially resolved prior to inclusion. Poorly performed prosthetics may potentially contribute to peri-implant attachment loss by e.g., obstructed measures of oral hygiene. Traumatic occlusion as a contributing factor needs further research. Animal experimental findings show that traumatic occlusal overload aggravate bone loss at implants with ligature induced infection.[185, 186] Since it is not clearly demonstrated that traumatic

occlusion does not contribute to peri-implant bone loss, occlusal correction should be performed prior to final inclusion.

(4) The implant site may have been grafted prior to implant placement. This must be elucidated on prior to treatment of a peri-implant defect since the biological situation may be totally different if there are remaining old graft particles in the surrounding bone.

(5) Angulations of the implants and potentially partial placement outside of the skeletal envelope may be detrimental to reconstructive treatment. This needs to be explored prior to therapy.

(6) Reason for tooth loss may vary from patient to patient. It is not resolved if patients with a history of periodontitis respond as well to treatment of peri-implantitis as compared to periodontaly healthy patients. The same issue may have an impact on the outcome of regenerative or reconstructive measures. It is also sometimes not known if the patient has a history of chronic or aggressive periodontitis and if these two disease entities affect the outcome of peri-implantits therapy.

(7) As of today there is a great range of implant systems on the market. There have been major changes of all implants systems throughout the years. Implant development take place both at the macroscopic as well as the microscopic level, i.e., with both new prosthetic connections, implant body design, such as thread types, as well as surface characteristics down to the nano level. Before including an implant such factors should probably be defined and potentially later balanced between the test and control group. By experience it may sometimes be extremely difficult to track what exact implant the referred patient has. Even if a specific surgical approach works for one type of implant it may well be that a different implant may respond less favorable.

(8) Implants with a one stage installation protocol may be difficult to re-submerge in conjunction with surgical therapy of peri-implant osseous defects. Early penetration of the implant through the mucosa may potentially affect the outcome of the therapy.

(9) Presence or absence of keratinized mucosa may potentially affect the outcome of a therapeutic approach. In study IV a weak positive correlation with r =0.371 between presence of keratinized mucosa and no progression of bone loss was found.

Only subjects with removable and preferably screw retained supraconstructions were included in the study presented in paper IV. The reasons for this were:

(1) The possibility to remove the supraconstruction and thus perform more accurate clinical measurements.

(2) To gain access for the surgery.

(3) Since a submerged protocol was used, removal of the supraconstruction was obviously mandatory.

(4) To check potential prosthetic complications such as loose abutment screws potentially contributing to the etiology. A complete prosthetic assessment is only possible after removal of the supraconstruction. One example of this is fractured bridge screws or fractured implant walls hidden under a full implant retained bridge.

(5) Resonance frequency analysis (RFA) can only be performed at the implant level.

(6) To exclude loose implants hidden under full implant retained bridges it is also mandatory to remove the supraconstructions.

(7) To avoid excess dental cement as a contributing factor to the peri-implantitis lesion.[187]

In paper IV the patients were given a combination regime of antibiotics i.e., Amoxicillin and Metronidazole. There is no scientific evidence for the adjunctive use of antibiotics when

performing surgical corrective treatment of peri-implant osseous defects, but anecdotal evidence seems to support its use. Opposed to periodontal treatment where the antibiotic when used, is usually an adjunct to the causative treatment phase, it has been a convention to instead use antibiotics in conjunction with surgery when treating peri-implantitis. The reason is probably that it has been considered impossible to perform sufficiently good closed implant surface debridement and disruption of the biofilm since access is often difficult. Leonhard et al.[188] reported that the typical periodontal pathogens Porphyromonas gingivalis, Prevotella intermedia and Aggregatibacter actinomycetemcomitans were recovered from the peri-implant sulci in 60% of patients which may support the adjunctive use of Amoxicillin and Metronidazole.[189-191] It is however important to point out that the microbiological diversity is much higher in the peri-implantitis lesions and atypical oral microorganism, such as Staphylococcus Epidermidis[188] are more frequently found in peri-implantitis lesions than in periodontitis lesions. The general understanding is though that the peri-implantitis subgingival microbiota is a mixed anaerobic infection dominated by gram negative bacteria i.e., a flora similar to chronic periodontitis.[192] A standardized antibiotic regime was used for all patients in an attempt to avoid having to control for differences in antibiotics used when analyzing the results. One alternative may potentially have been to perform microbiological testing and try to level out the microbiota prior to surgical intervention but there is no support for such a regime in the literature.

After mechanical debridement of the implants a chemical surface cleansing with 24% ethylenediaminetetraacetic acid (EDTA) -gel was performed. There is limited scientific evidence in the literature for usage of chemical agents for decontamination of dental implants. The choice to use 24% EDTA-gel was done on arbitrary means and it has never been shown that it has any positive clinical effects in conjunction with treatment of peri-implantitis, nor in conjunction with surgical treatment of periodontal defects.[193, 194] EDTA is a pH neutral

calcium chelator which has been reported to dissolve the smear layer of organic and mineralized debris, consisting of bacterial plaque and contaminated cementum and dental calculus left after root surface debridement.[195] Importantly and in contrast to many other chemical decontaminants, EDTA-gel has been shown to have no necrotizing effect on the surrounding tissues.[196] It was judged to be of significant importance to maintain as much as possible of the vitality of the surrounding tissues, primarily due to the potential for bleeding to the defects hence rapidly achieving a stable blood coagulum. The major biological concept behind utilizing titanium as a biomaterial for reconstructive therapy lies in the thrombogenic effects and from this perspective it was decided to use EDTA-gel as a chemical "decontaminant." Later in vitro results from our department have demonstrated that EDTA has no significant effect on the biofilm of a titanium surface and with this more recent knowledge in mind it may have been preferable to choose a chemical decontaminant with better biofilm distruptive potential such as e.g., citric acid or H_2O_2.[197]

No selection was made based on the defect morphology. With respect to defect width and defect angle a clinical judgement was done after surgical exploration of the defect and potential for accomplishing graft stability. It was later shown that the groups were balanced with respect to number of defect walls and defect angle. A recent study report that the defect angle has a significant effect on the potential to achieve regeneration of a peri-implant osseous defect[198] and the results may well have been different if the study was purified to narrower or only 3 wall defects leading to improved stability of the inserted PTG particles. In this context one must though keep in mind that many peri-implant defects are rather wide.

The clinical study presented in paper V was executed to get an initial evaluation of the performance of PTG when used in degree II furcation defects and to achieve pilot data and gain experience with the methodology prior to initition of a full randomized clinical trial. All

patients included clearly had a history of chronic periodontitis and had undergone a causative periodontal treatment phase. It may though well be that some of the teeth had undisclosed contributing etiological factors such as root fractures, partial pulpal necrosis with lateral canals invading in to the furcation or such, but the fact that the results were relatively homogenous probably opposes this. The teeth were all vital and the defects were limited to the furcations. The proximal bone height was above the level of the furcation entrance in four of the included teeth while six of the included teeth had the proximal bone height at the level of the furcation entrance.

A number of factors seem to influence the outcome of the surgical reconstructive therapy of furcation defects such as:

(1) Vitality of the involved tooth.

(2) Traumatic occlusion.

(3) Height of the root trunk.

(4) Furcation defect morphology with e.g., respect to presence or absence of a buccal bony wall.

(5) Divergence between roots.

(6) Morphology of the root cones with sometimes deep concavities on the distal surface of the mesial root hindering proper instrumentation prior to grafting.

(7) Height of the buccal keratinized gingiva affecting the risk of recession of the gingival margins and exposure of the grafted furcation.

(8) Patient related factors such as smoking.[199]

The study presented in paper V did not involve sufficient number of patients to control for such variables and it may be that the outcome of the study would have been different under other and potentially more optimal conditions.

Inclusion criteria however left many teeth or subjects out from participation based on the above described variables. Attempts were also made to optimize the potential for a successful outcome in all treated defects both with respect to tooth related factors such as removal of enamel pearls and aiming at the best possible surgical technique.

Analytical methods

The study presented in paper II was designed to evaluate new bone formation along the root surfaces and histological sections were intended to be cut out at the bucco-palatal midline of the teeth. It was though established that mini pig teeth have some characteristics that makes it difficult to evaluate all aspects of bone growth in furcations:

(1) Concerns were raised with respect to remaining bone due to incomplete instrumentation of the pronounced mesial and distal radicular grooves. This made it difficult to clearly determine if bone along the root surfaces above the notch was newly formed or remaining old bone which had been overlooked while creating the defects. Due to this it was decided to solely analyze vertical bone formation at the mesio-distal center of the furcations. It is also important to point out that this concern also may be related to other animal models that have been used for studies on regeneration of furcation defects. It has previously been reported that osseous regeneration in furcations first occur adjacent to the root surfaces followed by fill of the more central portion of the inter-radicular area.[200] Such a verdict may obviously be related to the here mentioned root morphological findings. It is also important to point out that the presence of radicular grooves differs to a large extent between species. Further evaluation of animal model related issues is important and microCT is a valuable tool to perform such investigations. One may also consider to use methods for labeling new bone such as injection with fluorochromes during healing.

(2) The root dentin of the mini pigs has a low degree of mineralization and is sometimes difficult to clinically differentiate from bone. This makes preparation of the defects relatively complicated and perforation in to the pulpal space excludes a tooth from analysis.

(3) The premolar roots of the mini pigs are rather slender and curved. MicroCT images were utilized to direct the histological sectioning, but it was still sometimes difficult to embrace the complete surfaces of both the mesial and the distal roots in the same histological section.

The bone regeneration rate in mini pigs has been reported to be relatively similar as for humans.[177, 201] The bone regeneration rate in pigs has been reported to be 1.2 to 1.5 mm/day as compared with humans which is one to 1.5 mm/day.[202] One may with this in mind argue that a longer period of healing would have given a different outcome of study II. The literature on growth rate in the jaws of mini pigs is however scarce and unclear and I feel that further studies would be motivated to resolve this issue. The experience from our lab is that in alveolar bone of mini pigs growth is significantly faster than in humans and has in our previous experimental studies been judged to be approximately twice as fast. The exact growth rate needs though to be addressed in a separate animal investigation which specifically is designed to look at growth kinetics of bone defects. From this perspective it may potentially be interesting to instead use a circular critical size defect model in alveolar bone.[174]

The statistical comparisons both in study I and II were performed on a defect level. The New Zealand white rabbits are, as are laboratory animals in general, systemically inbreeds, which means that the genetic variation between animals is highly reduced. In addition to this the uniformity and consistency of the environmental setting and animal housing makes

comparisons at a defect level feasible. This consideration also relates to the mini pigs in paper II.

In paper I and II the new bone was analyzed both by microCT and histology. The advantage with the microCT analysis as compared to histology is the three dimensional mode of analysis which is accomplished by non destructive microCT technique. The marrow compartment of the tibia defects obviously lacked osseous boundaries and the spread of the granules thus varied between the defects. Analyzing new bone within a distinct volume of interest (VOI) was hence not possible to accomplish for the marrow compartment section of the defects, i.e., to define comparable volumes in both tests and control groups was not doable. Therefore, it was decided to analyze bone within a VOI solely in the cortical compartment of the defects (Fig. 11).

A cylindrical VOI, 2.8 mm in diameter and 46 slices in height (i.e., 0.67 mm) i.e., a total volume of 4.13 mm^3), in the center of the critical-size defect and excluding the walls consisting of old bone was created. The microCT analysis software was then used to distinguish between air filled spaces, titanium and bone, setting the upper grey threshold value to 110 and setting the lower grey threshold value to 60.

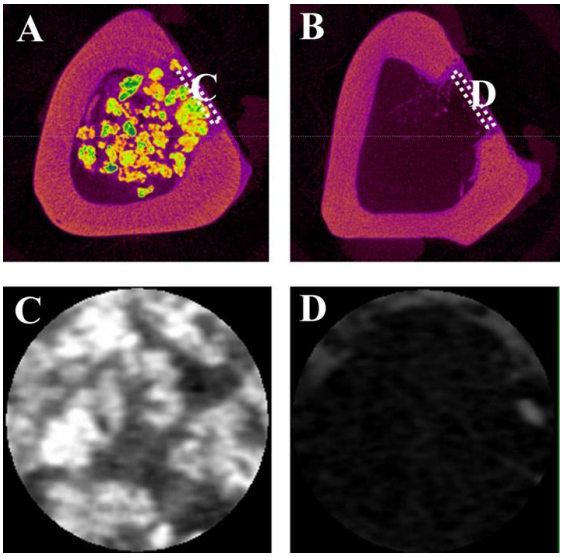

Figure 11. Paper I: MicroCT analyzes of bone within a volume of 4.13 mm^3 in the cortical compartment of the defects A, C) PTG, B, D) Sham

The new bone volume within the VOI was compared between test and control defects. One may argue that the solely cortical VOI limits the value of the microCT analysis which in part is why a more extensive histological analysis was performed.

It is important to point out that the grey threshold values for bone were derived from the cortical old bone. More immature woven bone may thus have been omitted from the analysis. Importantly, precise quantitative measurements of the bone volume are difficult because of scatter effects from the metal particles which makes the interface between the biomaterial and new bone imprecise. Attempts were made to perform a virtual eradication of the granules but due to their irregular architecture this was at the time of the analysis not possible.

A general consideration for all non digital analyses performed is the problem with blinding the examiner due to the metallic, non-resorbable properties of PTG. This consideration is reflected both in the histological analyses and microCT analyses in study I and II as well as in the radiographic analyses of the clinical studies. With respect to the clinical analyses in paper II, IV and V it was not possible to clinically distinguish between subjects based on presence of PTG due to the mucosa coverage of the bone substitute. The digital analysis using the microCT software to some extent resolves this potential bias consideration.

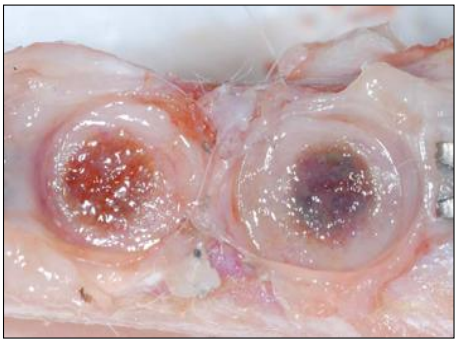

Figure 12. Tibia defect model used in study I: Clinical picture after removal of titanium implants and collection of wound fluid. Wound fluid was sampled with 6.25 mm circular filter papers. RNA was also isolated from the tissue that attached to the implant covering the defects and analyzed through real-time (RT)-PCR.

Wound fluid was sampled in conjunction with harvest at 4 weeks (Fig. 12).

The wound fluid samples were taken after removal of the titanium coins i.e., at the interface between the titanium coin and bone substitutes and new bone.

The capability to perform gene expression analyses in small samples of tissue is important for assessing the biological performance of new biomaterials in the field of regenerative surgery. Importantly the RT-PCR results are solely indicators for several biomarkers related to bone formation, resorption and inflammation, and should thus be interpreted accordingly. While the gene expression rate is presented as numbers of PCR cycles, corrected for the total amount of RNA and normalized for the relative values of several housekeeping genes, the significance of the results is sometimes difficult to interpret. The results show the trends in gene expression within the isolated tissues and this molecular data should be interpreted only together with the results from the histological and microCT analyses. One may thus argue that the results should not be related to the microCT and histological findings since sampling was made at a much more superficial level and that the titanium coin obviously also affects the outcome hence the wound fluid results is the joint outcome of effects from both PTG and the titanium coin surface. A more invasive method to sample wound fluid or tissue would however have destroyed the samples, and disqualified them from histological and microCT analyses.

The clinical measurement of the pigs at time of harvest was performed right after euthanasia. This may consequently have made the BoP recordings obscure. However, since all animals were treated in the same fashion, i.e., with probing directly after euthanasia, the significance of this is probably not a major concern. It would have been more stressful for the animals to undergo one additional sedation for performing the clinical measurements and since the value of the clinical results in this study still was considered to be limited this procedure was chosen for animal ethical reasons.

In paper III sections from the base of the defect to the top of the implant were analyzed both by microCT and histology. The implant was not notched at the base of the defect and the sectioning was done in a fashion perpendicular to the implant (i.e., horizontal sectioning, Fig. 13). Bone growth was demonstrated between the implant and the PTG particles to at least half way from the base to the top of the defect, where obviously no old bone was present (Fig. 13). The microCT and histological sections were compared with both clinical pictures as well as with intrasurgical registrations of the defect morphology.

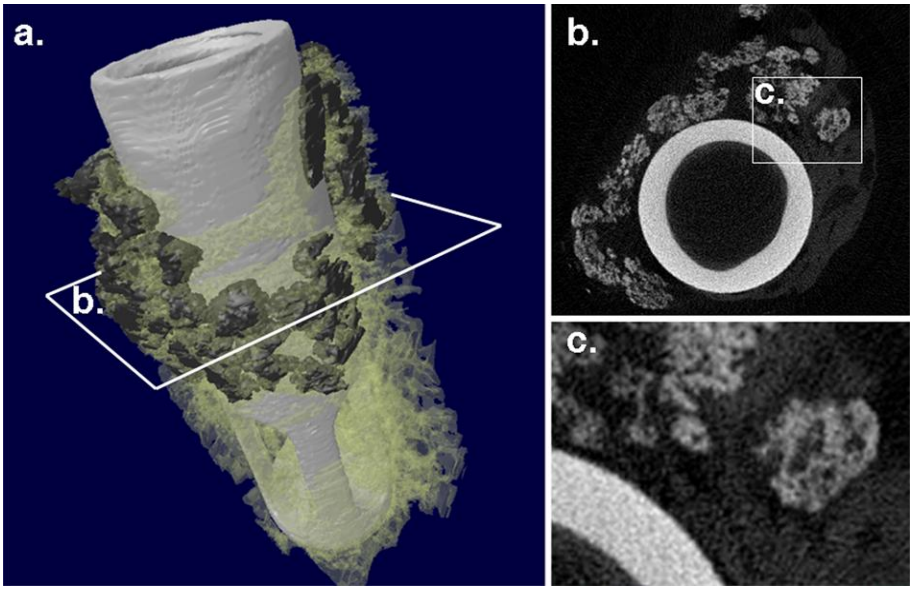

Figure 13. a) 3D reconstruction of microCT images of en bloc excised trephine biopsy containing implant, bone and PTG particles. b) Horizontal section at the vertical midpoint of the defect. c) Magnification of b with new bone embedding PTG particles as well as new bone in close contact with the implant surface implying re-osseointegration.

The aim with sectioning the implant in a horizontal fashion was to study ingrowth of PTG into bone. This was done both within the intraosseous component of the defect as well as facially of the fenestrated implant section, where the PTG particles were placed without the stability of bony walls. One may argue that arbitrary microCT threshold values for bone may have skewed the results in a positive fashion. Opposing to this is the fact that the threshold

value for bone was set with lower and upper values corresponding with the bone tissue at the base of the implant which hence was clearly defined as old bone. This may instead have led to a less positive outcome, since immature woven bone, with less degree of mineralization, as a consequence may have been omitted.

Attempts were made to analyze CAL in the study presented in paper IV. Many of the implants had been placed deep with a thick and bulky mucosa coronal of the implant and with manifest bleeding and inflammation at baseline. It was thus difficult to justify CAL or bone sounding measurements, since localization of a rigid reference point with high reproducibility was difficult to ascertain. Moreover, 12 of the 33 patients received new supraconstructions, which made baseline reference points for CAL or bone sounding measurements obscure at the later examination points. In hindsight, relating on a reference point at the coronal margin of the implant body was not an optimal approach for CAL measurements. Using the margin of a healing abutment or even fabricating a stent type device with vertical canals, for accurate and reproducible measurement at the different examination time points may have made CAL measurements possible with acceptable accuracy. One may also argue that CAL measurements under all circumstances are of less value, due to the non-resorbable nature of PTG.

No significant difference in PPD between the test and control groups was demonstrated. Probing of the peri-implant mucosal crevice to disclose clinical signs of inflammation and loss of bone are important for diagnosing peri-implantitis, but PPD as a surrogate marker for assessing the outcome of reconstructive peri-implant therapy, call for discussion. It has been reported that factors such as mucositis, applied probing force and accessibility to the pocket have an impact on the penetration of the periodontal probe.[203-207]

Lang and co-workers[206] compared probe penetration, as seen on histological sections, with true attachment level in healthy and inflamed peri-implant tissues. An error of up to 1.6 mm at implants with peri-implantitis was reported. The error was smaller at healthy sites or at sites with only mucositis and instead only varied between -0.35 and +0.3 mm. To compensate for such errors a "click-on" probe with a defined probing force (20 g) was used in study IV (Fig. 14).

A recently published systemic review discussed endpoints, which have been used in studies on peri-implantitis treatment.[208] These authors argued that the most valid clinical outcome in such studies would be implant loss. PPD and CAL are non validated surrogate markers for disease progression and it has never really been shown that a demonstrated change in CAL or PPD will capture the risk for future loss of an implant. The patient group in study IV will be followed carefully over time to reveal implant loss in the case or control group.

From the perspective of reconstructive outcomes, bone fill is the only component of the potentially regenerated peri-implant tissues in a treated peri-implant defect that can be rigidly assessed clinically. With the implant *in situ*, true validation of bone fill can only be done by re-entry surgery.[209] In view of patient morbidity, re-entry surgery is often impractical to execute. As a consequence to this, radiographic assessments, as well as clinical variables, such as CAL and bone sounding, have been implemented as surrogate markers to bone fill.

BoP and PLI was scored dichotomously in the study presented in paper IV. No difference between groups was found. It may have been preferable to use a graded index such as the modified bleeding index (mBI).[150] The obvious crux with BoP was to use it as a surrogate marker for presence or absence of active disease. It is from this perspective, thus argued that a dichotomous scoring is more rigid.

In hindsight an intra-examiner calibration prior to initiation of the study should have been performed. On the other hand a proper calibration should if so have been performed in peri-implantitis sites with at least a 5 days interval between probings.[210] This would have meant that the supraconstruction should have been removed a second time, which would be difficult to ethically justify. Furthermore, a defined force "click-on" periodontal probe was used and probing was performed at six sites around each implant (Fig. 14).

Figure 14. 0.20 N (20 g) defined force "click-on" periodontal probe (University of North Carolina, DB 764 R, AESCULAP®, B Braun, Tuttlingen, Germany)

Figure 15. Resonance frequency analysis using the Osstell mentor® (Osstell AB, Gothenburg, Sweden) with the SmartPeg™ inserted into the implant and directing the Osstell® probe against the peg at four different directions to achieve the lowest ISQ value which then was recorded.

When initiating the study, it was to the best of our knowledge the first time a non-resorbable bone substitute was used in a case-control clinical study setting for reconstructive treatment of peri-implant osseous defects. It was consequently not possible to derive data on power from another study to determine the number of patients needed. With the aim to develop this novel surgical methodology and to calculate power, a small pilot study was performed prior to study start. Resonance Frequency analysis (RFA) (Fig. 15) was at the time of the present study initiation considered to be an objective way to quantify implant stability[211] and judged to be a potential surrogate marker for

osseointegration.[212]

The manufacturer of the RFA claims that one millimeter of peri-implant marginal bone loss, correspond with a loss of three ISQ. Based on this, one millimeter of re-osseointegration would reasonably correspond with a gain of three ISQ. Zarb and Albrektsson[212] suggested a clinically applicable (i.e., non-destructive) definition of osseointegration instead based on implant stability: "a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading." In this context RFA may be a potential candidate for a non-invasive objective method to assess implant stability and bone to implant contact.[211] Measuring RFA was consequently decided to be done in an attempt to determine change in implant stability between baseline and the 12 months follow-up. Later studies have though questioned the rigidity of RFA and a recently published animal experimental study report no correlation between histological assessment of osseointegration measured as bone to implant contact (BIC) and RFA values.[213] The rigidity of RFA to be used as an instrument for the analysis of defect fill of peri-implant osseous defects and potentially re-osseointegration remains however to be determined.

It is of significant interest to find objective non-destructive parameters for osseointegration. The results from this study do not provide any clear evidence in either direction and further studies will be needed.

Clearly the fact that titanium is metallic is a hindrance from the perspective of radiographic evaluation of bone fill and the non-resorbable nature of PTG made discrimination between true re-osseointegration or true bone fill of the defects and integrated PTG particles impossible. It may thus be argued that the results from this study have limited value. On the contrary to this, it is argued that this study has clinical relevance since: (1) Importantly both

treatment modalities lead to significant reductions in both pocket depths and radiographic defect height at 12 months as compared with the baseline measurements. This may indicate that either one of these two methods may have some potential in the treatment of peri-implant osseous defects. Very few randomized case control clinical studies have so far been published evaluating regenerative treatment of peri-implant osseous defects, thus such findings are valuable from a clinical perspective. There was a tendency towards less defect progression and improvement in RFA in the test group but longer observation times will be necessary (Table 2). The patients in paper IV are being followed and comparisons between groups at 3 and 5 years will be performed. No adverse events were seen for the PTG treated defects which is important to know when planning for further studies on this novel biomaterial.

	PPD				**Radiographic defect height**			
	Case		**Control**		**Case**		**Control**	
mm change	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
1.5-	0	0	2	12.5	0	0	3	18.75
>0 - 1.5	0	0	1	6.25	2	12.5	4	25
0	5	31.25	2	12.5	0	0	0	0
<0- -1.5	0	0	2	12.5	4	25	7	43.75
-1.5 -	11	68.75	9	56.25	10	62.5	2	12.5
Total	16	100	16	100	16	100	16	100

Table 2. Study IV: Frequency distribution. Change in PPD (p =0.17) and radiographic defect height (p <0.01) selecting on the mesial or distal defect with the deepest infrabony component as recorded during surgery. X^2-test.

In paper V the main endpoint for validating the performance of the material was change in vertical and horizontal bone sounding, which would indicate defect fill, albeit it do not provide true evidence for integrated PTG particles, in-growth of bone or regeneration of

periodontal attachment. Invasive evaluation methods would be necessary to fulfill such aims with the study. The non-resorbable metallic thus radiopaque nature of PTG makes the radiographic endpoint obscure for showing true defect fill. One would also tend to expect that the non-resorbable nature of PTG would tend to hinder probe penetration and thus by all means lead to reduction of clinical measurements of defect width and height. The outcome in paper V was on the contrary opposite to this, which hypothetically implies that the biomaterial was embedded in loose connective tissue which makes it possible to penetrate the periodontal probe between the PTG particles. To further elucidate on this a re-entry surgery will be necessary. There were even examples of defect progression but, since no control group were included it was not possible to determine if this finding was caused by the material per se or from bone remodeling due to the surgical trauma.

Statistical considerations

The study presented in paper I included a total of 15 separate tests and the outcome of the analysis should be considered explorative rather than giving a definite answer to one specific hypothesis. It should thus be noted that a number of the positive findings in study I were as a matter of fact rejected when Bonferroni correction where applied. In lieu of this one may argue that each analysis of the different biological markers may be considered as a separate experiment, which accordingly make Bonferroni corrections unnecessary, i.e., to avoid false rejection of the alternate hypothesis.[214, 215]

Since data in paper II was pooled from three sets of surgery series, it was decided to further elucidate on potential differences between the three experimental surgical time points. The sham results were compared between the three series of surgeries. A Kruskal-Wallis test demonstrated no statistically significant differences between outcome of sham treatment in the three animal experimental surgeries ($p > 0.05$). Statistical comparisons were performed at

the tooth site level. When comparing the distribution of teeth P2, P3 and P4 between groups in paper II, no significant difference was seen (p =0.64). Furthermore a Pearson product-moment correlation coefficient was calculated in the study presented in paper II, comparing two separate histo-morphometrical readings by the same examiner (SPL) with an interval of two months. A positive correlation between the two separate histological readings by the same examiner (blinded from results of the first reading) with r =0.567 was demonstrated. In the clinical study presented in paper IV it was not possible to blind the examiner when performing the radiographic measurement. The radiographs were however randomized with respect to time-point, i.e., the examiner was blinded to at which examination time-point the specific radiograph was taken. Radiographs from the first 19 subjects were analyzed twice with an interval of two weeks. Pearson correlation coefficient was calculated comparing the two separate radiographic readings, demonstrating a strong positive correlation between two separate readings, with r =0.996. The clinical study presented in study IV compare a case and control group of patients, whereas no control group was included in the study presented in paper V. This is an obvious limitation with paper V. Baseline data compared with 12 months data is instead presented.

Discussion of results

This series of studies evaluate titanium when used as non-resorbable bone substitute and its efficacy for reconstructing some types of osseous defects. The initial animal experimental studies were encouraging and led to the execution of two clinical studies. The choices of clinical surgical indications were peri-implant osseous defects and degree II mandibular furcation defects. Both these indications are complex and numerous suggestions have been made throughout the years to find potential treatment methods. As of today there are no predictable evidence based methods for treating neither peri-implant osseous defects nor

furcation defects. It is thus considered to be of significant interest to evolve on these indications.

In paper I it was by histological means observed that mineralized tissue form directly onto the titanium granules. Mineralized tissue was also filling the voids between the titanium particles. This observation verify that porous titanium granules are osteoconductive, which correspond with previous findings as reported by Turner et al.[129] It is interesting that new bone had formed on PTG particles residing deep in the marrow compartment and without obvious connection to the old cortical bone. Since the tibial bone in rabbits does not exhibit trabecular bone structures in the bone marrow space the histological observation of trabecular bone growth within the marrow compartment would thus potentially suggest that the titanium granules act as an osteoconductive scaffold also when placed in the bone marrow. This newly formed bone may:

(1) origin from the old cortical bone and grow along the surfaces of the titanium granules into the subjacent marrow compartment.

(2) be formed by bone apposition directly onto the implant surface a.k.a. contact osteogenesis.[62]

If the latter bone forming mechanism is factual, it is a crucial observation in study I. However further verification in experiments, specifically focusing on the growth kinetics of the involved tissues, will be necessary. This bone formation is also interesting, since one might suspect that the granules within the marrow space were not completely immobile during the healing phase.Still bone has formed in close connection with the particles. It may furthermore be interesting to elucidate on the porosities of the granules which from this perspective is a protected environment which potentially may favor osseous growth.

A suggested cause for orthopedic hip replacement femoral stem, implant loosening, is the biological reaction to wear particles from the actual implant. It has been demonstrated that

such wear particles induces a granulomatous reaction, with subsequent release of cytokines, which potentially may induce bone resorption.[216-218] It has also been shown that tissues proximal to failed prosthetic implants, demonstrate copious macrophages and foreign-body giant cells as well as profuse particulate wear debris.[217, 219, 220] An *in vitro* experimental study demonstrated that exposure to titanium-vanadium-aluminum particles can induce increased release of lactate dehydrogenase (LDH), which is a sensitive marker for tissue necrosis.[221] Small metal particles have been demonstrated within the cytoplasm of macrophages while larger metal particles seem to be encapsulated by connective tissue with a mild or no signs of an inflammatory infiltrate. The particle diameter of PTG is 700-1000 μm, whereas the experiments referred to above, studied particles less than one micrometer in diameter. Since the heat oxidized PTG (i.e., WPTG) is brittle, the risk of fragmentation must though be kept in mind from the perspective of a foreign body reaction, which obviously may have fatal consequences when the material is used for augmentation purposes. It was thus decided to further elucidate on this and in study I it was decided to evaluate a range of markers potentially indicative of necrosis, inflammation and bone resorption. No significant difference in LDH activity between test and control defects was found, which supposedly would indicate that the PTG and WPTG particles do not induce tissue necrosis. To further study this potential issue, smaller fragments of the PTG and WPTG particles should be employed in similar test systems.

An in vitro study compared leukocyte activation on titanium surfaces with different surface properties such as oxide layer thickness. It was reported that after four hours of incubation in blood, implants with a thick oxide layer had fewer monocytes residing on the surface than implants with a thin oxide layer.[116] Such findings shall not be put in direct comparison with the findings from paper I but defects treated with the heat oxidized WPTG particles showed significantly less total protein when compared to PTG and sham, but significantly higher

collagen-I mRNA levels. Based on the findings in paper I, one may hypothesize that the degree of oxidization may have an impact on inflammatory mechanisms, albeit one would then tend to expect significant differences between WPTG, PTG and the control group for the IL-6 and IL-10 mRNA levels. It is not realistic to conclude on these early observations but to further elucidate on potential anti-inflammatory mechanisms of TiO_2 is of significant interest.

Paper II suggested that implantation of experimental degree II furcation defects with PTG led to significantly better osseous defect resolution as compared with DBBM implanted sites. The comparisons between PTG and sham were though incoherent as two out of four of the microCT analyzes as well as the histomorphometrical comparisons did not show significant differences between groups. A vast number of previous animal experimental studies have been performed with the aim to study various methods for grafting furcation defects. Murakami et al.[222] performed animal experimental studies in monkeys and beagle dogs to test the regenerative potential of basic fibroblast growth factor in a gelatinous carrier. Furcation defects 4 mm in height and 3 mm in width, i.e., significantly smaller than in paper II, were created and filled with either the test material, the carrier alone or left empty. The healing time was 6 weeks for dogs and 8 weeks for monkeys. Significantly better healing was seen for the test group as compared to the control group with respect to mean percent of the area with newly formed bone. For the test group, 79.6 % new bone was demonstrated in the dogs and 71.3% new bone was demonstrated in the monkeys. It is not possible to make a direct comparison with the results from study II, but a mean vertical osseous formation of 62.9% on the mean was seen for PTG treated sites as demonstrated with histology and 84.4% with microCT. In an animal experimental study in monkeys, executed by Ripamonti et al.,[223] significantly better bone height regeneration was seen for defects treated with BMP-2 alone as compared to recombinant human osteogenic protein (OP-1) with or without BMP-2. Insoluble collagenous bone matrix was used as a carrier in all groups. The healing time was 60 days. A

mean regenerated vertical bone height of 48.3 % was seen for the BMP-2 alone group. Takayama et al.[224] evaluated regenerative treatment of degree II furcations with recombinant basic fibroblast growth factor (FGF) using a primate model. The healing time was eight weeks. 71.3% new vertical bone height was seen for the FGF treated defects.

The 62.9% mean regenerated bone height in paper II corresponds well with historical results (Appendix I) but it is also important to keep in mind that PTG is a non-resorbable material. Albeit bone was analyzed as new bone between PTG particles it is still not completely comparable to a situation where the biomaterial is completely resorbed as the PTG particles may act as a permament growth scaffold for the bone and potentially hinder resorption. Moreover also the sham defects demonstrated good results with a mean new vertical bone height of 61.5%, which was not significantly different than the PTG treated defect. It is also important to point out that many of the historical controls employed smaller defects and with a longer healing time. Such factors may have an impact on the results and direct comparisons between studies should thus be avoided.

In the human histological analysis presented in paper III it was shown that PTG integrate well in human alveolar bone and this study supports the principle that re-ossseointegration of a dental implant is feasible. To the best of my knowledge this is the first published human histological data showing re-osseointegration of an ailing dental implant. The histological findings concur with findings by Alfram et al.,[128] but these authors evaluated the performance of PTG when used for stabilizing a titanium hip stem implant in conjunction with the primary surgery. From the perspective of infection of the surgical site the intraoral situation is also much more intricate as compared to the closed environment of a hip implant.

In a prospective randomized clinical trial, Deppe et al.[225] performed treatment of 73 peri-implant defects in 32 patients. Four different surgical methods including airpowder abrasive and CO_2 laser for decontamination of implants, and a combination of an alloplast bone

substitute, consisting of β-tricalcium phosfate mixed with autogenous bone and GTR, were studied. Patients were followed between five and 59 months. For the grafted sites, without GTR, a reduction in PD of 2.3 mm was shown. This corresponds relatively well with the findings in paper IV. A prospective randomized parallel arm clinical study by Khoury and Bouchman[226] evaluated grafting of peri-implant defects with autogenous bone with or without GTR. 41 implants in 25 patients were included. Implants were submerged after the surgical therapy. A reduction in PD at the three years follow-up of 5.1 mm for the defects treated with the graft alone was seen. The radiographic defect height for the defect treated with the bone substitute alone was reduced with 2.4 mm on the mean. In a case control clinical study of 12 months duration Roos-Jansåker et al.[105] analyzed reconstructive treatment of peri-implant osseous defects with a coralline xenograft with or without GTR. No significant differences between groups were shown and a reduction in PD of 3.4 mm for the defects treated solely with the bone substitute i.e., without GTR was found at the 12 months evaluation. With respect to radiographic defect fill a gain of 1.4 mm was seen for the defects filled with the bone substitute alone. These implants were left non-submerged after the surgical treatment whereas the implants treated in paper IV were left submerged for a period of 6 months. This may to the least hypothetically be the reason for the difference in the PD reduction between the Roos-Jansåker study as compared with paper IV in the sense that the submersion may have lead to a coronal positioning of the flap margin. A prospective, randomized, parallel arm, clinical study by Schwarz et al.[227] compared grafting of peri-implant defects with either nanocrystaline HAp or DBBM. A resorbable collagen membrane was also used in the DBBM group. Implants were left non-submerged after surgical intervention. A reduction in PD for the implants sites treated with nanocrystaline HAp of 1.5 mm was shown at the two year follow-up whereas the DBBM and GTR treated sites demonstrated a reduction of 2.4 mm.

Recently, the four year follow-up data from the same study was presented with stable PD reductions.[228]

The demonstrated radiographic defect fill in PTG treated sites correspond relatively well with the findings in the studies by Khoury and Buchmann[226] and Roos-Jansåker et al.[105] It is noteworthy that both these independent investigations report that the additional utilization of GTR did not lead to significant additional improvement in neither clinical nor radiographic parameters in comparison with grafting alone. In the current study, PTG was used without GTR. At some occasions dislodged PTG particles were seen, especially in wider defects. A combination of PTG and a titanium reinforced GTR or a titanium mesh may from this perspective be interesting to investigate in future studies.

With respect to fill of osseous peri-implant defects PTG may have a potential, but to evaluate true endpoints such as progression of bone loss, longer follow-up time is necessary. It is essential to follow this patient group over time to evaluate the long-term outcome of grafting with PTG as compared with the control group.

In paper V the performance of PTG when used as bone substitute in the surgical treatment mandibular degree II furcations was analyzed. In a systematic review by Reynolds and co-workers[229] on the usage of bone replacement grafts in the treatment of periodontal osseous defects it was stated that with regard to degree II furcations, clinical results after the application of bone grafts or bone graft substitutes were superior to open flap debridement alone. Aimetti et al.[230] analyzed a combination of enamel matrix derivative (EMD) and autogenous bone grafts in the treatment of mandibular degree II furcation defects. After 24 months of follow-up the mean vertical attachment level at bone sounding was reduced with 3.6 mm and the horizontal attachment level at bone sounding was reduced with 3.4 mm. It has also been reported that the combination of a bone replacement graft with GTR may be beneficial. A recent study by Santana and collaborators[231] investigated the combination of

HAp mixed with tetracycline and GTR for treatment of mandibular degree II furcations. After 12 months the mean improvement of the vertical clinical attachment level was 3.1 mm whereas the horizontal attachment level gain was 3.5 mm on the mean. (Appendix III)

General conclusion

Both the animal experimental and human histological results suggested that PTG is biocompatible and can promote healing of osseous defects and re-osseointegration of dental implants. The clinical results were intriguing, but inconclusive. The general null hypothesis was only in part rejected. Further studies, combining PTG with other reconstructive therapies such as GTR, or potentially used as a carrier for bone growth promoting molecules, is encouraged.

Conclusions related to specific aims

Within the limitations of this thesis, it was demonstrated that:

A) Both PTG and WPTG are osteoconductive graft materials suitable for regenerative treatment of osseous defects. (*Paper I*)

B) PTG integrate well in newly formed bone and can also be used safely adjacent to titanium implants and without inducing bone resorption or inflammation. However to address the efficacy of PTG and WPTG when utilized as graft material in osseous regenerative surgery adjacent to functionally loaded titanium implants, clinical trials will be needed. (*Paper1*)

C) Based on the findings in paper II it is suggested that PTG may integrate well in alveolar bone and supports osseous re-growth in degree II furcation defects. PTG led to significantly better defect resolution than treatment with DBBM or sham. The comparisons between PTG and sham were, however, inconsistent as two of the four microCT analyses, as well as the histomorphometrical comparisons, did not demonstrate significant differences. (*Paper II*)

D) The findings presented in paper II support that PTG is safe to use in close proximity to root surfaces. Some signs of regenerated PDL were demonstrated. Regarding presence

of root resorption lacunae no significant difference between groups were found. (*Paper II*)

E) The human histological analysis presented in paper III suggests that PTG integrate well and promote re-osseointegration of dental implants placed in human jaw bone. (*Paper III*)

F) The findings presented in paper IV show that reconstruction with PTG lead to significantly better radiographic peri-implant defect fill compared with controls. These results do not necessarily imply re-osseointegration or osseointegrated PTG particles. Improvements in clinical parameters were observed in both the case and control group, however no significant differences between the groups were demonstrated. (*Paper IV*)

G) It is suggested that PTG is safe to use in close proximity to root surfaces of mandibular molar furcations but no significant improvements in clinical endpoints of defect resolution were observed in this case series. (*Paper V*)

Future perspectives

The ultimate outcome of osseous reconstructive therapy is complete regeneration of bone and the gold standard is the utilization of autogenous bone grafts. Degradation of the graft material and substitution with new bone is considered to be the optimal outcome after a grafting procedure, but the importance of this is not completely clear. It may well be that a bone substitute, that do not resorb, is favorable for some indications, since osseous healing and maturation is a biological process extensive in time.

As more implants are used to replace missing teeth an escalating number of individuals will potentially be affected by peri-implantitis. Avoiding progression of bone loss is an important goal for peri-implantitis treatment but reconstruction of lost peri-implant bone is of significant interest in the aesthetic zone and in other areas where osseous resective surgical techniques are judged inappropriate (Fig. 16) from a technical perspective, such as proximity to neighboring teeth or implants. By this it is indisputable that new surgical strategies for treating peri-implant osseous defects are important to develop. Even if it has been suggested that the problem with peri-implant osseous defects can be surgically resolved with an apically positioned flap type surgery, the main predicament with such an approach is again a peri-implant osseous defect approximating a neighboring tooth. To make it possible to apically position the flap, it will in such cases be necessary to remove alveolar bone and attachment on the tooth (Fig. 16). The dilemma with such an approach is obvious. Again with respect to implants in the esthetic zone with peri-implant defects, it may be difficult to motivate the patient to denude the implant body to gain access for oral hygiene measures.

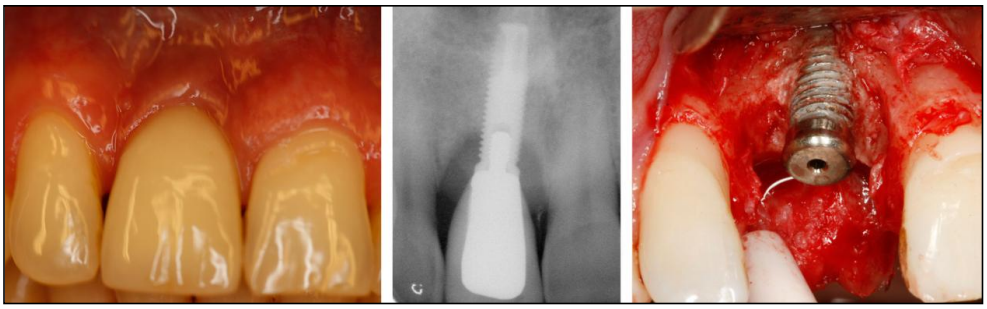

Figure 16. Clinical pictures and radiograph of implant regio 11 (FDA) with a peri-implant osseous defect. Note the close proximity of the osseous defect walls to the neighboring teeth excluding the possibility of treatment with an apically positioned flap with osseous resection. Removal of the implant would create a major osseous defect making esthetic reconstruction challenging. The value of further evolving within the field of regenerative therapy is obvious

While more and more researchers present data on the prevalence of peri-implantitis, dental implant scientists has in recent years, changed from being purely focused on developing the implants per se, towards instead finding methods to preserve ailing implants. The importance of this, both from the perspective of the affected patients, as well as for the therapists is obvious. One must though not forget that much of the complications with resulting extensive and costly treatments, potentially might have been avoided, if the teeth had been saved in the first place. From this perspective doing science on osseous reconstructive biomaterials is of utmost importance.

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Errata

On page 40 row 9-11 should be changed to: “Bucco-palatal cylindrical microCT cores demonstrated a median defect fill of 96.8% for PTG-implanted defects, which was significantly greater than sham (72.2%) and DBBM (62.0%) (P <0.001) treatments.”

On page 52 row 10 after the word “defects” three references has fallen out:
Roussa E. Anatomic characteristics of the furcation and root surfaces of molar teeth and their significance in the clinical management of marginal periodontitis. *Clin Anat* 1998;11:177-186
and Novaes AB, Jr., Palioto DB, de Andrade PF, Marchesan JT. Regeneration of class II furcation defects: determinants of increased success. *Braz Dent J* 2005;16:87-97
and reference number 199.

Reference 62 (Hanser) is mistakenly inserted and shall be omitted and replaced by reference number 61 (Davies JE, 1998)

Paper I: Abstract: reactuion should be changed to reaction

Paper I page 172: Column one last sentence now reads “These findings can clearly not be put in direct comparison with our data, but is interesting to note that the defects treated with WPTG demonstrate significantly less total protein when compared to PTG and WPTG” and should be changed to: “These findings can clearly not be put in direct comparison with our data, but is interesting to note that the defects treated with WPTG demonstrate significantly less total protein when compared to PTG and sham.”

I

II

III

IV

Appendix I

Author, year	Materials and methods	Treatment groups (n)	Endpoints	Results
Wikesjö et al. (1988)	14 beagle dogs Surgically created defects + 6 weeks without plaque control + intervention + harvest at 12 weeks	4 groups: Citric acid, +/- fibronectin Tetracycline hydrochloride +/- fibronectin	2 groups Group 1: 8 animals, 47 sites Group 2: 6 animals, 36 sites	1) Citric acid or tetracycline hydrochloride resulted in frequent complete connective tissue repair 2) No significant difference connective tissue repair and root resorption and ankylosis
Van Swol et al. (1989)	4 stump tail monkeys, Surgically created defects + immediate intervention + harvest at 6 weeks, 12 weeks, 18 weeks and 24 weeks	Randomized to defect fill with: 1. Amalgam 2. Zink oxyphosfate cement 3. Glass ionomer 4. Control untreated defect	n =16 sites, Only an observational study and no statistical analysis performed.	Clinical, radiographic and histological results showed that glas ionomer was well tolerated and biocompatible. Inflammation was seen at amalgam and zink oxyphosfate cement sites
Caffesse et al. 1990	6 beagle dogs, Naturally occurring periodontitis. SRP + 1 month later intervention + membrane removal at 4 weeks + harvest at 3 months.	Randomization 1.Test = OFD + GTR (ePTFE, Gore Tex) 2. Control = OFD alone	n =6 dog with 8 teeth per dog Statistical unit =animal	Significantly better regeneration of connective tissue attachment and bone in GTR group.
Caffesse et al. (1993)	4 beagle dogs, Naturally occurring periodontitis. SRP + 1 months later intervention + membrane removal at 6 weeks + harvest at 5.5 months	Randomized to: 1. Demineralized, freeze-dried human cortical bone grafts (DFDCB) + GTR (ePTFE) 2. GTR (ePTFE) alone	4 dogs with 4 + 4 teeth (mandibular P2, P2, P4 and M1) Analysis at tooth level within dog Histology	No significant difference in bone, cementum or connective tissue between groups. Mean new bone DFDCB + GTR: 35.1% (area). Mean new bone GTR alone: 35.2% (area).
Plotzke et al. (1993)	4 beagle dogs, Surgically created defects + immediate intervention + harvest at 4 months	Randomization 1. Non-resorbable calcium-layered polymer of polymethyl-methacrylate and hydroxyethyl-methacrylate (HTR) 2. Sham	Histology, Statistical unit = animal N =6 teeth each dog, 5 dogs =30 defects	HTR was well tolerated and acted as a biocompatible "filler." Regeneration of bone was significantly better in control defects. HTR 30.8% new bone (area) Control 72.1% new bone (area)

Lekovic et al. 1993	6 mongrel dogs. Naturally occurring periodontitis. SRP + 1 month hygiene phase+ intervention + GTR removed at 4 months + harvest at 6 months.	Randomization Porous calcium phosphate granules in all test defects+ 1. GTR: Polycarbonate filter (Millipore) 2. GTR: Silicone rubber 3. GTR: Expanded polytetrafluorethylene (ePTFE, Gore-Tex) 4. GTR: Polycaprolactone 5. Sham	n =5 furcations in each dog	All test groups significantly better than control and no significant difference between the 4 tests. New bone:Test groups: 1.74-2.02 mm, Control. 0.34 mm All test groups significantly better than control and no significant difference between the 4 tests. Polycaprolactone and Polycarbonate had more inflammation than control. No root resorbtion or ankylosis.
Dyer et al. (1993)	8 beagle dogs, Naturally occurring periodontitis. SRP + 1 months later intervention+ membrane removal at 6 weeks + harvest at 4 months	Randomized to: 1.Tetracycline HCl+ GTR Expanded polytetrafluorethylene (ePTFE) 2. Citric acid + GTR (ePTFE) 3. GTR (ePTFE) alone	Histology n =12 quadrants, 48 teeth	Regeneration of cementum, PDL and bone in all groups. No significant difference between groups
Caffesse et al. (1994)	Nine fox hound dogs (periodontally healthy) SRP + plaque control + after 2 weeks Surgically created defects+Intervention + Harvest at 1 months, 3 months and 6 months	Designated to ePTFE (GoreTex) vs. 2 bioabsorbable membranes made from a synthetic copolymer of glycolide and lactide (Resolut)	n =9 dogs with 4 defects per dog but 3 timepoints i.e., 12 defects per time point and 3 treatment groups (i.e., n =3 + 3 + 3 per time point) Histology Mean per tooth within animals	No significant difference in connective tissue attachment, cementum, bone or epithelial downgrowth between groups at any time point. 6 months data: ePTFE mean % new bone height: 59.6 Resolut type 1 mean % new bone height: 66.3 Resolut type 2 mean % new bone height: 76.6
Ripamonti et al. (1994)	3 baboons- SRP+ plaque control program until clinically free of gingivitis- Surgically created defects + intervention + 5 months healing + harvest	Designated to 1.Bovine BMP + BMP3 in insosoluble collagenous bone matrix (BMP-ICBM) 2. ICBM alone	Histology n =6 + 6 teeth	Significantly better regeneration of bone and periodontal attachment in BMPs-ICBM group as compared to ICBM alone. Approximately 51% new bone height in group BMPs+ ICBM as compared to 24% for ICBM alone.

Vernino et al. 1995 Rajnay et al. (1997) Butler et al.(1998)	6 baboons, Surgically created defects + Twisted wire with gause 3 weeks + 10 weeks undisturbed healing + surgical intervention- harvest one animal at 6 weeks and 5 animals at 13 weeks	Randomized to: 1.Open flap debridement (OFD) + GTR (ePTFE, Gore-Tex) (n =15) 2. OFD+GTR (polylactic acid, Epi-Guide) (n =14) 3.OFD alone (n =5) 4. Surgery alone i.e., SRP (n =5)	Statistical unit: Furcations n =39 Comment: Performed a Pearsons correlation coefficient analysis to check within animal response. (1 animal with only test 5 animals with test and control)	No significant difference between groups. OFD alone had the most overall defect fill.
Polson et al. (1995)	6 beagle dogs, surgically created and naturally occurring defects. Immediate intervention- Reentry at 4-5 months, harvest at 9-12 months Mechanical and chemical oral hygiene during healing phase	Designated to 1.GTR (Polymer of lactic acid (PLA) dissolved in N-methyl-2-pyrrolidine (NMP), Atrisorb) No control	Histology 11 surgically induced defects and 4 naturally occurring defects. Unclear if only furcation defects were included.	Reentry: Surgically induced defects: 100 % new bone in furcations Naturally occurring defects: 100% new bone in furcations Surgically induced defects: 72% of root surface had new connective tissue attachment Naturally occurring defects: 77% of root surface had new connective tissue attachment
Ripamonti et al. (1996)	3 baboons Model according to Ripamonti et al. 1994 but only 60 days of healing	Designated to 1.Recombinanat human osteogenic protein-1,100 µg (hOP-1) in bovine insosoluble collagenous bone matrix (**b**ICBM) (n =4) 2. Recombinanat human osteogenic protein-1, 500 µg 100 (hOP-1) in bICBM (n =6) 3. bICBM alone (n =2)	Statistical unit: Defects n =12	No significant difference between the two different concentrations of hOP-1 but both hOP-1 groups significantly better than control (bICBM-alone) .

Giannobile et al. (1996)	10 Cynomolgus monkeys, Ligature (3.0 silk) induced periodontitis-When established lesions after 12 – 16 weeks: SRP- oral hygiene 3 weeks-Surgery 1(3 months specimen) – at 2 months surgery 2 (1 months specimen)- harvest 3 monstha after surgery 1	Randomized to: 1.Insulin like growth factor-1 (IGF-1), 10 µg 2. Platelet derived growth factor (PDGF), 10 µg 3. PDGF/ IGF 4. Control: methyl-cellulose vehicle alone	Statistical unit: quadrants n: One animal excluded, 2 quadrants each animal designated as control 27 quadrants test i.e., 9 quadrants each group.	IGF-1 vs control: no difference PDGF significantly better than control PDGF+ IGF-1 significantly better that PDGF alone
Denesh-Meyer et al (1997)	11 sheep, Surgically created defects + immediate intervention+ harvest at 7 weeks	Designated to 1.GTR GoreTex periodontal material 2.GTR Soft Tissue pack 3.Sham	Histology	Significantly better regeneration of bone, cementum and connective tissue attachment in GTR groups No difference between GTR groups
Hurzeler et al 1997	Orthodontic elastics induced defects (Caton & Zander 1975)-5 months-Stainless steel wire extending into the buccal furcations+ plaque accumulation for 3 months- wires pushed further into furcations every other day-thereafter SRP + plaque control regimen for 3 weeks-then surgical intervention- 5 months healing time-harvest	Designated to: 1.GTR Bioresorbable copolymer of glycolide and lactide, Millipore) 2.Sham	Histology	Significantly better regeneration of bone, cementum and connective tissue attachment in GTR groups
Bogle et al. (1997)	6 beagle dogs, Naturally occurring periodontitis, Split mouth, Intervention + oral hygiene + harvest at 6 months.	Designated to: 1. Test: Atrisorb (n =8) 2. Control: Sham (n =8)	Statistical unit: animals n =6	Significantly more bone and cementum but not connective tissue attachment in test sites

Cirelli et al. (1997)	4 mongrel dogs, Surgically created defects + 8 weeks days of healing with gutta perka in defects + intervention + harvest at 3 months	Randomized to: 1. Test: OFD+ GTR Bovine collagen (n = 8) 2. Control: OFD (n = 8)	Statistical unit =teeth n =14 teeth (2 excluded) Control =6, Test =8	Significantly less epithelial down groth and more cementum in GTR group but no significant difference in osseous regeneration
Lekovic et al. (1998)	7 mongrel dogs. Naturally occurring periodontitis. 1 month hygiene phase+ intervention+ harvest at 6 months.	Randomized to: 1. GTR: Polycarbonate filter (Millipore) 2. GTR: Silicone rubber 3. GTR: Expanded polytetrafluorethylene (ePTFE , Gore-Tex) 4. GTR: Polycaprolactone 5. Sham	Statistical unit =teeth n =5 teeth in each dog, 7 dogs n =35 i.e., n =7 + 7 + 7 + 7 + 7	1) Significantly more cementum and osseous regeneration in all test groups as compared with sham. 2) Significantly more inflammation in Polycarbonate filter and polycaprolactone groups.
Mohammed et al. (1998)	24 Sheep Surgically created defects but with 2 weeks of ligature prior to intervention-intervention- Harvest after 2 or 6 weeks	Randomized to: 1.OFD+ carrier el (25% pluronic F127) only 2. OFD+TGF-β in carrier gel+ GTR (ePTFE, GoreTex) 3. OFD+ TGF-β in carrier gel alone	24 sheep 48 defects Statistical unit =teeth 24 teeth each time point	Significantly more bone in TGF-β sites and TGF-β+ GTR sites vs control and significantly more bone in GTR+ TGF-β vs TGF-β alone. No differences in cementum formation.
Murakami et al. (1999)	6 Beagle dogs and 4 Macaca fascicularis monkeys Surgically created defects + one month with impression material in defects thereafter surgical interventions Dogs sacrificed at 6 weeks and monkeys after 8 weeks	Designated to: 1.OFD + gelatinous carrier alone 2. OFD + gelatinous carrier with recombinant basic fibroblast growth factor (bFGF)	Histology Statistical unit: teeth Beagle dogs: 4 controls and 7 test Monkeys: 10 controls and 6 test	bFGF was well tolerated and acted as a biocompatible "filler". Regeneration of bone was significantly better in control defects. Test: Dogs: 79.6 % new bone Monkeys: 71.3% (area)

Ripamonti et al., (2001)	3 baboons, Model according to Ripamonti et al. 1994 but only 60 days of healing	Designated to: 1.Recombinanat human osteogenic protein-1,100 µg (OP-1) in insoluble collagenous bone matrix (ICBM) (n = 2) 2. OP-1 100 µg in ICBM + BMP-2 100 µg n = 6 3. ICBM+BMP-2 100 µg n=4	n =4 defects per animal 3 animals 12 defects	BMP-2 alone significantly better bone regeneration than OP-1/BMP-2 combined and OP-1 alone. BMP-2: 48.3% new bone (mean height) OP-1: 38.6% new bone (mean height). OP-1/BMP-2: 40.5% new bone (mean height).
Takayama et al. (2001)	4 Macaca fascicularis monkeys, Surgically created defects + one month with impression material in defects thereafter surgical interventions Monkeys sacrificed after 8 weeks	Designated to: 1.OFD + gelatinous carrier alone (n =10) 2. OFD + gelatinous carrier with recombinant 0.1%basic fibroblast growth factor (FGF-2) (n = 8) 3. OFD+ gelatinous carrier with recombinant 0.4 % FGF -2 (n =6) 4. No treatment (n =8)	Statistical unit: teeth n =8 teeth each monkey i.e., 32 teeth	Significantly more new bone and cementum in FGF-2 0.4 % vs. control. Significantly more new cementum but not bone in FGF-2 0.1 % vs. control. FGF-2 0.4%: 71.3% new bone FGF-2 0.1 %: 58.0% new bone
Dogan et al. 2002.	One dog, Surgically created furcations cell seeded with biopsy bone harvested from two ePTFE treated surgically created furcation in the same dog. Harvest at 42 days	1. Cell seeded furcations 2. Controls left untreated (sham)	n =4 mandibular furcations (2 test and 2 control) No statistical analysis performed	More new bone, cementum and connective tissue attachment in cell seeding group. Mean new bone in cell seeding group: 51.2% (area). Mean new bone in control: 32.9% (area).
Cetiner et al., 2004	4 mongrel dogs. Surgically created defects + immediate intervention + harvest at 7 months	Randomly assigned to: 1. GTR PLA: Liquid polymer membranes (LPM, Atrisorb®) 2. GTR PLA: Resorbable periodontal mesh (RPM, Resolut®)	n =10+10 defects	No significant difference in new attachment and new bone between groups.

Regazzini et al. 2004	4 mongrel dogs. Hygiene phase 2 weeks prior to experiment- Surgically created defects + gutta percha for 3 weeks- intervention + GTR removed at 4 weeks + harvest at 8 weeks	1. OFD + EDTA gel 2. OFD + EDTA gel + EMD OFD+ EDTA gel + EMD+ GTR (GoreTex)	n =7 (control) + 8 + 8.	Significantly more bone in EMD alone group as compared with the EMD + GTR and sham. Mean new bone in sham: 21.7% (area). Mean new bone EMD: 28.5% (area). Mean new bone EMD+ GTR: 67.4% (area) No difference in cementum formation between groups.
Nagai et al. (2005)	8 beagle dogs, Surgically created defects + 4 weeks with impression material in defects thereafter removal of sponge + oral hygiene for 2 weeks then surgical interventions Dogs sacrificed after 4 or 12 weeks	1. Platelet-derived factor releasate (PR) on collagen sponge 2. Collagen sponge alone 3. Control (lingual aspect of each defect)	n =3 + 3 mandibular defects each dog, 8 dogs, 48 defects	Significantly more cementum and bone in PR defects vs. control or collagen sponge alone. More new bone in both sponge alone and PR sites than control. Mean new bone in PR: 38.6% (area). Mean new bone collagen sponge: 27.4% (area). Mean new bone control 3.7% (area)
Miranda et al. (2006)	3 beagle dogs, surgically created defect+ impression material in defects for 21 days+ 21 days healing + experimental surgery- GTR removed at day 30- harvest at 4 months	1.GTR of modified glas ionomer cement (GIC) 2.GTR Polylactic acid (GUI) 3.Control	n =9 (3 + 3 + 3) teeth No statistical analysis performed (too small n)	Median new bone in GIC: 20.6% (area). Median new bone GUI: 54.3% (area). Median new bone control: 24.6% (area) AN! Median values
Macedo et al. (2006)	6 mongrel dogs. surgically created defects+ impression material in defects for 21 days thereafter SRP + 2 weeks of healin+ Experimental surgery+ harvest at 12 weeks.	Randomized to: 1. GTR ePTFE (GoreTex) with membrane removal at 2 weeks. 2. GTR ePTFE (GoreTex) with membrane removal at 4 weeks	2 + 2 teeth each dog Analysis at animal level n =6	No significant difference in new bone, new cementum or new connective tissue between groups. Mean new bone in 2 weeks GTR removal: 66.7% (area). Mean new bone in 4 weeks GTR removal: 70.5% (area).
Deliberador et al. (2006)	6 mongrel dogs, Profylaxis one week prior to Surgically created defects. Immediately treated- harvest at 90 days.	Randomized to: 1.Sham (blood clot alone) 2. Autogenous bone (AB) 3. AB+ GTR: Calcium sulphate (CS)	6 teeth/ dog n =12 + 12 + 12	No significant difference between groups, i.e., sham as good as autogenous + GTR or autogenous alone. Mean new bone in sham: 61.9% (area). Mean new bone in autogenous bone alone: 65.0% (area). Mean new bone in autogenous bone + GTR: 59.9% (area).

Christgau et al. (2007)	21 beagle dogs. Naturally occurring periodontitis but surgically standardized defects+ OFD + immediately treated+ Harvest at 2, 4 and 8 weeks (4 dogs each) + 3, 6 and 12 months (3 dogs each)	Randomized to: 1.GTR Polydiaxonon (Mempol) 2. GTR Polylactic acid (Guidor) 3. GTR Collagen (Biogide) 4. GTR (ePTFE, GoreTex) 5. Non randomized control (tooth P3)	Total 126 teeth, 6 time points 2, 4, 8 weeks: 4 teeth per group per time point and control 8 teeth per time point. 3, 6 and 9 months: 3 teeth per group per time point and control 6 teeth per time point.	Immuno histochemical and histological study on periodontal wound healing dynamics after GTR treatment of furcations.
deAndrade et al. (2007)	6 mongrel dogs. 2 weeks profylaxis followed by surgically created defects+ impression material in defects for 2 weeks and thereafter SRP + 2 weeks of healing + Experimental surgery + harvest at 12 weeks.	Randomized to: 1.Control: OFD+GTR Polyglycolic acid: trimethylene carbonate (PGA:TMC) 2. Test: OFD+GTR Acellular dermal matrix (ADM)	2 + 2 teeth each dog, Analysis at animal level n =6	ADM resulted in greater increase in keratinized gingival otherwise no significant differences between groups Mean new bone in ADM: 70.3% (area). Mean new bone in 4 weeks PGA:TMC: 55.1% (area).
Teare et al. (2008)	4 Chacma baboons, heteroptopically induced bone formation in rectus abdominus muscle + surgically created defects -healed 40 days- treatment- Harvest at 60 days	1.Matrigel® alone (control) 2. TGF-β3+Matrigel® 3. TGF-β3+ Matrigel® induced bone (IB) 4. Matrigel® IB 5. TGF-β3+osteogenic protein-1 IB+ ICBM	n =16	TGF-β3 IB + Matrigel® IB significantly more bone than control. Mean new bone TGF-β3+Matrigel® IB: 58.9% (area). Mean new bone Matrigel® IB: 64.9% (area). Mean new bone control: 31.3% (area)
Morris et al. 2008	Seven beagle dogs, furcations surgically created+Twisted wire with gause-12 weeks-defects re-cut if remodeled + treatment + Injections repeated weekly for 3 weeks – harvest after 12 weeks	Randomized to: 1.OFD+Methylcellolose gel (control) (n=7) 2. OFD+Methylcellolose gel+ 0.5 mg Simvastatin (test 1, n=3) 3. OFD+Methylcellolose gel+ 2.0 mg Simvastatin (test 2, n=4)	2 teeth each dog	No difference in inflammation. Greater remaining bone loss in 0.5 mg Simvastatin group than control. No difference in remaining bone loss between 2 mg Simvastatin and control.

Keles et al. 2009	4 mongrel dogs, oral hygiene phase- Surgically created defects (5mm in height and 2 mm in depth) + 3 weeks with impression material in defects thereafter removal of sponge + oral hygiene for 2 weeks then surgical interventions- harvest at 12 weeks	1.OFD + platelet rich plasma pellet (PP) + GTR Atrisorb. 2. OFD + PP 3. OFD alone	n =24 (8 + 8 + 8)	More cementum in group PRP + GTR and PRP alone vs control group No significant difference in new bone between groups 1and 2 but both test groups were better than the negative control. Mean new bone in PR+ GTR:61.1% (area). Mean new bone PP alone: 62.6% (area). Mean new bone control: 42.4% (area)
Simsek et al. (2010)	3 mongrel dogs + surgically created defects + impression material for 3 weeks + SRP+ after 2 weeks of plaque control: surgical intervention + harvest at 8 weeks	1. OFD 2. OFD + PRP 3. OFD+ autogenous bone (ACB) 4. OFD+ ACB + PRP 5. OFD+ mesenchymal stem cell (MSCs) + PRP	Comparisons at defect level (n =6 per group)	No significant difference in alveolar bone fill between groups but significantly more cementum formation in group 3, 4, 5 as compared to group 1.
Suaid et al. (2011)	Surgically created defects, split mouth immediately treated and harvest after 3 months	Collagen sponge +/- seeding with autologous PDL cells with Collagen GTR (Resolut) over all defects	7 dogs, 14 defects Histology	Significantly more new cementum, new bone and new PDL and significantly less down-growth of epithelium in GTR + PDL seeded defects as compared to GTR alone

Appendix I. Animal experimental studies on reconstructive treatment of degree II furcation defects. Pubmed database were searched with the following terms and key words and limited to animals: furcation (total hits 250). Furthermore the reference lists in the publications listed were manually searched for additional references. Searches were limited to animals.

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Appendix II

Author, year	Study design, n, implant type	Antibiotics	Surface decontamination	Treatment groups (n)	Endpoints	Results
Bach et al. (2000)	Prospective non-randomized parallel arm clinical study n =30 patients Implant type not specified	Not reported	Decontamination with diode laser 20 seconds at each implant.	1) Control: Initial therapy, resective phase, reconstructive phase with osseous augmentation as needed, recall phase 2) Test: Same as 1 but with diode laser decontamination for 20 seconds of each implant at the resective, reconstructive and at all recall appointments.	Probing depths (PD) Bleeding on Probing (BoP) Radiographs Microbiology (DNA-RNA-hybridization probes)	**Baseline:** PD >5 mm, radiological evidence of bone loss otherwise not specified. **Very limited data and no statistics reported and insufficient results supplied to support any conclusions.** Relapse rate (time point not specified) (%): Test: 11, Control: 34 **5 years survival rate:** Survival rate (%): Test: 100, Control: 99
Khoury and Buchman (2001)	Prospective non-randomized parallel arm clinical study of 3 years duration. n =25 patients, 41 implants IMZ, Frialit	According to susceptility testing in conjunction with non-surgical causative treatment and 4 weeks prior to surgery and at surgery + 7 days.	A combination of 0.2% chlorhexidine digluconate, Citric Acid (pH =1) for 1 minute, H_20_2 (concentration not specified) and irrigation with sterile saline Instrument type not reported	1) Autogenous bone graft alone + submersion (FG, n =7). 2) Autogenous bone graft + non-resorbable GTR + submersion (FGRM n =7). 3) Autogenous bone graft + bioresorbable GTR+ submersion (FGM, n =11).	PD Probing bone levels (BL) Radiographic defect height (DH) Implant mobility with Periotest (PT)	**Baseline:** mean PD (mm): 8.0, mean BL (mm): 7.5 **3 years (change from baseline, mean values):** Change in PD (mm): FG: - 5.1, FGRM: -2.6, FGM: -5.4 FGM and FG significantly better than FGRM ($P \leq 0.05$) Change in BL (mm): FG: -3.2, FGRM: -2.3, FGM: -3.4 No significant difference between groups Change in DH (mm): FG: -2.4, FGRM: -1.9, FGM: -2.8 No significant difference between groups Change in PT (mm): FG: -1.7, FGRM: -0.5, FGM: -2.0 No significant difference between groups

Romeo et al. (2005, 2007)	Prospective randomized case control clinical study of 2 and 3 years† duration. n =17 (19*) patients, 35 implants with 24 solid screws and 11 hollow screws all Straumann TPS.	Amoxicillin 50 mg/ kg/ day for 8 days in conjunction with causative treatment	Metronidazole gel (Elyzol®) and a solution of Tetracyclin hydrochloride for 3 min and irrigation with sterile saline Instrument type not reported	1) Test: Apically positioned flap with resective surgery + Implantoplasty (n =10) 2) Control: Apically positioned flap with resective Resective surgery alone (n =7 No information on submersion but supraconstructions seemingly removed (clinical picures)	Modified sulcus bleding index (mBI) PD Pseudopockets (DIM) Mucosal recession (REC) Probing attachment level (PAL) Radiographic marginal bone loss (MBL) Survivalrate	**Baseline:** mean PD (mm): 5.8, mean PAL(mm): 5.5 **2 years† (mean values at evaluation):** mBI: Test: 0.5 , Control: 2.3 which was significantly lower in test group PPD (mm): Test: 3.58 , Control: 5.5 which was significantly lower in test group REC (mm): Test: 2.3 , Control: 1.64 which was significantly lower in control group PAL (mm): Test: 5.89 , Control: 7.04 which was significantly lower in test group **2 years (mean values at evaluation):** MBL (mm): Test: 3.89, Control: 4.46 which was significantly lower in test group **3 years (mean values at evaluation):** MBL (mm): Test: 3.88, Control: 5.39 which was significantly lower in test group **3 years survival rate:** Survival rate (%): Test: 100, Control: 77.8 which was significantly lower in control group.
Deppe et al. (2007)	Prospective non-randomized parallel arm clinical study n = 32 patients, 73 implants (IMZ, Frailit, Brånemark, Straumann screw type)	Not given at any time point	Air-powder abrasive (Prophy-Jet) CO_2 laser decontamination	G1) Air-powder abrasive + soft tissue resection (n =6) G2) Air-powder abrasive + alloplast +autologeous bone (n =7) + submersion 4 months G3) Air-powder abrasive (Prophy-Jet) + CO_2 laser decontamination +	Sulcus bleeding index (SBI) PD Distance implant-mucosa (DIM) Clinical attachment level (AL) Radiographic distance implant to bone(DIB) (on OPGs) on some implants	**Baseline:** mean PD (mm): 5.3, mean AL(mm): 6.5 **Follow up between 5 and 59 months (mean 37 months)** Comparisons in between resection groups (G1 and G3) and in between augmented groups. **Lost implants** G1 = 3, G2 =4, G3 =2, G4 =4 **SBI:** No significant difference between groups **Mean change in PD between surgery and final evaluation (mm)** G1 =-0.8, G2 =-2.3, G3 =-2.7, G4 =-2.5 Statistical analysis not reported

Deppe continued				soft tissue resection (n=10) G4) Air-powder abrasive + CO_2 laser decontamination + alloplast (Pure-Phase β-Tricalcium Phosfate , Cerasorb)+ non-resorbable GTR‡ +autologeous bone (n = 9) + submersion 4 months		**Mean change in DIM between surgery and final evaluation (mm)** G1 =0.8, G2 =0.2, G3 =2.4, G4 =-0.2 Statistical analysis not reported **Mean change in AL between surgery and final evaluation (mm)** G1 =0.0, G2 =-2.1, G3=-0.3, G4=-2.7 G1 significantly better than. G3 P <0.05 G2 vs. G4 N.S. **Mean change in DIB between surgery and final evaluation (mm)** G1 =0.3, G2 =-2.1, G3 =-0.4, G4 =-2.2 G1 significantly better than G3 P <0.05 G2 vs. G4 N.S.
Roos-Jansaker et al. (2007)	Prospective non-randomized case control clinical study n =36 patients, 65 implants (63 Brånemark, 2 AstraTech)	Amoxicillin (375 mg X 3) and Metronidazole 400 mg X 2) for 10 days Initiated the day before surgery	H_20_2 3% + irrigation with saline. Titanium curettes	1) Test:Xenograft (Algipore®) + resorbable membrane No submersion of implants but supraconstructions removed at surgery n =29 2) Control: Xenograft alone n = 36 Non-submerged healing	PD Probing attachment level (PAL) Mucosal recession (MR) Bleeding index (BI) Bleeding on probing (BOP) Radiographic defect fill	**Baseline:** mean PD (mm): 5.5, mean PAL (mm): 7.0 **1 year:** Reduction in PD (deepest site, mm): Test: 2.86, Control: 3.44 No significant difference between groups Gain in PAL (deepest site, mm): Test: 1.59,Control: 1.8 No significant difference between groups MR (deepest site, mm): Test: 1.28, Control: 1.61 No significant difference between groups Reduction in BI: No significant difference between groups BOP (% of sites +): Test: 22, Control: 25 No significant difference between groups Radiographic defect fill (mean of m and d site, mm): Test: 1.52,Control: 1.44 No significant difference between groups.
Schwarz et al. (2009, 2008)	Prospective randomized parallel arm	Not reported	Plastic curettes+ sterile saline	1) Test: Access flap + nanocrystaline	BoP PD MR	**Baseline:** mean PD (mm): 7.0 , mean CAL (mm): 7.4 3 patients excluded

Schwarz continued	clinical study n = 22 patients Brånemark, camlog, Straumann cylindrical screw, KSI baure Schraube, MTX Spline twist. Tapered Screw Vent, ZL-Duraplant,			hydroxyapataite (NHA, Ostim) Non-submerged healing 2) Control: natural bone mineral (BioOss) + collagen membrane (BioGide) (NBM + CM) Non-submerged healing	CAL Radiographic analysis	**2 years:** Reduction in PD (deepest site, mm): Test:1.5, Control: 2.4 Gain in CAL (deepest site, mm): Test:1.0 ,Control:2.0 MR: Test: + 0.5, Control: +0.4 BOP (% of sites +): Test: 44, Control: 34 Radiographic analysis: A decrease translucency seen for both groups. No numerical data reported. Statistic analysis not reported for any of the parameters **4 years:** Reduction in PD (deepest site, mm): Test:1.1, Control: 2.5 Gain in CAL (deepest site, mm): Test: 0.6 ,Control: 2.0 MR: Test: + 0.4, Control: +0.5 BOP (% of sites +): Test: 48, Control: 28 Radiographic analysis: Increased (n =6)+decrease translucency (n =13)seen for groups. No numerical data reported. Statistic analysis not reported for any of the parameters

Appendix II. Prospective parallel arm clinical studies on surgical treatment of osseous defects caused by peri-implantitis or peri-implant osseous defects with undefined etiology. Pubmed database were searched with the following terms and key words: peri-implantitis OR peri-implant OR periimplantitis OR periimplant AND treatment (total hits 228) Searches were limited to humans. Furthermore the reference lists in reviews on surgical treatment of peri-implantitis were manually searched for additional references. * 17 patients analyzed clinically (Romeo et al. 2005) and 19 patients analyzed radiographically (Romeo et al. 2007). † No clinical comparisons were performed at 3 years. ‡ 2 of 10 implants treated only with GTR i.e., no alloplast

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Appendix III

Author, year	Materials and methods, n, follow up time	Treatment groups (n)	Results, mean gain PD, CAL, Hor
Pontoriero et al. (1987)	Randomized split mouth case control clinical trial (RCT), 37 patients, 6 months	OFD +/- GTR (Teflon, Gortex membrane)	Frequency of closed defects: 14/21 closed in test group versus 2/21 in control group
Becker et al. (1988)	Case series, 27 patients, 6 months	OFD + GTR (Teflon, Gortex membrane)	PD reduction: 2.5 mm Change in CAL: 1.3 mm Reentry defect depth reduction: 1.8 mm
Pontoriero et al. (1988)	RCT split mouth, 12 patients, 6 months	OFD +/- GTR (Teflon, Gortex membrane)	GI: Test: 0/11 sites at 6 months, Control: 2/12 sites at 6 month Reduction PD: Test 4.5 mm, Control 2.8 mm ($P < 0.001$) Improvement Probing attachment level (PAL)-Vertical: Test: 4.1 mm, Control: 1.5 mm ($P < 0.001$) Improvement PAL-Horizontal: Test: 4.1 mm , Control: 1.9 mm ($P < 0.001$)
Kenney (1988)	RCT split mouth, 23 patients, 6 months	OFD +/- porous hydroxyapatite (HA) implant (Interpore)	PD change: Test: (HA): 2.1 mm, Control: 0.57 mm (N.S.) CAL change: Test: (HA): 1.82 mm, Control: -0.04 mm ($P <0.001$) Vertical bone change reentry: Test: (HA): 1.95 mm, Control: -0.31 mm ($P <0.001$) Horizontal bone change reentry: Test: (HA): 1.56 mm, Control: -0.26 mm ($P <0.001$)
Gantes (1988)	Case control parallel arm clinical trial, 22 patients 30 defects, 12 months	OFD+ citric acid root conditioning +/- DFDBA graft. All flaps coronally positioned.	PD change: Test: (DFDBA): 1.6 mm, Control: 1.3 mm (N.S.) CAL change: Test: (DFDBA): 1.5 mm, Control: 1.6 mm (N.S.) Vertical bone change reentry: Test: (DFDBA): 2.4 mm, Control: 2.4 mm (N.S.) Horizontal bone change reentry: Test: (DFDBA): 3.0 mm, Control: 2.6 mm (N.S.)
Lekovic et al. (1989)	RCT split mouth, 21 patients, 6 months	OFD +/- GTR (Teflon, Gortex membrane)	Reduction PD: Test: 4.1 mm, Control: 1.1 mm Reduction GR: Test:-1.3 mm , Control:-1.1 mm Improvement CAL: Test: 2.9 mm, Control: -0.1 mm Reduction Bonesounding (BS) Vertical: Test: 0.2, Control: -0.2 Reduction BS horizontal: Test: 0.2 , Control: -0.1
Garrett, Martin and Egelberg (1990)	Parallel arm clinical study split mouth, 19 patients, 31 defects, 12 months	OFD + citric acid + DFDBA and Control: Coronal flap positioning (CF) or Test: dura mater membrane (DM)	Reduction PD: CF: 0.6 mm, DM: 1 mm, Mean defect fill: , CF: 70%, DM: 38% ($P <0.05$) Improvement in volume fill: CF: 16/16, DM 4/15 had a negative response Complete bony defect closure: CF: 9/16, DM: 3/ 15
Pepelassi et al. (1991)	RCT split mouth, 15 patients, 6 months	OFD +/- bone replacement graft of β-TCP, plaster of paris and doxycycline hyclate (test=grafted sites)	GI : N.S. Reduction Vertical -PD: Test: 2.3 mm, Control: 1.5 mm, N.S. Reduction Horizontal-PD: Test:1.1 mm, Control:3.7 mm ($P <0.05$) Improvement CAL: Test:1.9 mm , Control: 0.6 mm ($P <0.05$) Change vertical Vertical defect fill: Test: 2.7 mm, Control: 1.3 mm ($P <0.01$)

Anderegg et al. (1991)	RCT split mouth, 15 patients, 6 months	OFD + DFDBA +/- GTR e-PTFE (test =e-PTFE + DFDBA treated sites)	CAL- changes N.S. PD: Test: 3.1 mm Control:1.4 mm (P <0.05) Horizontal hard tissue change (re-entry): Test: 2.4 mm, Control: 1.0 mm (P <0.05) Vertical hard tissue change (re-entry) (CEJ-Base of defect): Test 3.5 mm, Control: 1.7 mm (P <0.05)
Flanary et al. (1991)	RCT split mouth, 19 patients, 6 months	OFD +/- synthetic wound dressing	PD change: Test:2.1 mm , Control: 1.2 mm (P <0.05) CAL change: Test: 1.0 mm , Control: 0.6 mm (P <0.05) Vertical re-entry change: Test: 1.0 mm, Control: 0.6 mm (P <0.05) Horizontal re-entry change: Test: 1.5 mm , Control: 0.8 mm (P <0.05)
Lekovic et al. (1991)	RCT split mouth, 15 patients, 6 months	OFD +/- CT graft	PD change: Test (CT graft): 4.14 mm, Control: 1.6 mm (P =0.001) CAL change: Test (CT graft): 2.4 mm, Control: -0.7 mm (N.R) Vertical bone probing change: Test (CT graft): 2.0 mm, Control: -1.27 mm (N.R) Horizontal bone probing change: Test (CT graft): 1.6 mm, Control: -0.2 mm (N.R)
Yukna (1992)	Prospective split mouth RCT, 11 patients with 22 defects, 12 months	OFD + rootconditioning with doxycycline paste and e-PTFE GTR or freeze dried dura mater allograft (FDDMA)	PD decrease: e-PTFE: 0 mm, FDDMA: 1 mm (N.S.) CAL gain: e-PTFE: 0 mm, FDDMA: 0 mm (N.S.) Horizontal furcation defect fill: e-PTFE: 1mm, FDDMA: 2 mm (P=0.04)
Quteish and Dolby (1992)	Prospective split mouth clinical trial, 19 patients, 52 defects, 6 months	OFD +/- collagen membrane (GTR)	PD change: Test: (GTR): 3.28 mm, Control: 2.12 mm (P <0.0001) CAL change: Test: (GTR): 2.73 mm, Control: 1.97 mm (P <0.001)
Parashis and Mitsis (1993)	Prospective RCT, 6 patients, 18 paired defecst , split mouth, 6 months	OFD + e-PTFE (GTR) +/- tetracycline root conditioning	PD change: Case (Tetracyclin): 2.4 mm, Control: 2.5 mm (N.S.) CAL change: Case (Tetracyclin): 1.6 mm, Control: 1.7 mm (N.S.)
Van Swol et al. (1993)	Prospective RCT Parallel arm case –control study, 38 patients, 3 months	OFD +/- Collagen GTR	PD change: Test (GTR): 2.14 mm, Control: 2.6 mm (N.S.) CAL change: Test (GTR): 1.41 mm , Control: 1.7 mm (N.S.) Horizontal bone probing change: Test (GTR): 2.28 mm, Control: 0.7 mm (P <0.001) Vertical bone change (reentry): Test (GTR): 1.68 mm, CT: 0.75 mm (P <0.05)
Bouchard, Ouhayoun and Egelberg (1993)	Prospective split mouth RCT, 12 patients with 24 defects, 12 months	OFD+ citric acid root conditioning and e-PTFE or connective tissue (CT) graft	PD change: e-PTFE: 2.8 mm, CT: 1.5mm CAL change: e-PTFE: 1.3 mm, CT: 1.2 mm Vertical bone change (reentry): e-PTFE: 0.4 mm, CT: 0.8 mm Horizontal bone change (reentry): e-PTFE: 2.2 mm, CT: 1.5 mm
Andersson et al. (1994)	Prospective split mouth RCT, 8 patients, 18 defects, 12 months	OFD and GTR (e-PTFE, Gore-Tex) or coronally positioned flap	PD change: Test: (GTR): 1.6 mm, Control:1.3 mm (N.S.) CAL change: Test: (GTR): 0.7 mm, Control: 0.4 mm (N.S.)

Wallace et al. (1994)	Prospective split mouth RCT, 6 patients, 17 defects, 6 months	OFD + e-PTFE +/- DFDBA	PD change: Test: (e-PTFE + DFDBA): 2.2 mm, Control (e-PTFE alone): 1.5 mm (N.S.) CAL change: Test: 0.8 mm, Control: -0.2 mm (N.S.) Vertical bone change reentry: Test: 5.0 mm, Control: 3.8 mm (N.S.) Horizontal bone change reentry: Test: 2.4 mm, Control: 2.3 mm (N.S.)
Yukna (1994)	Prospective split mouth RCT, 9 patients, 30 defects, 12 months	OFD + micropouros composite with calcium hydroxide (HTR) or autogenous osseous coagulum.	PD change: Test: (HTR): 2.1 mm, Control: 1.9 mm (N.S.) CAL change: Test: (HTR): 0.8 mm, Control: 1.0 mm (N.S.) Vertical bone change reentry: Test: (HTR): 1.6 mm, Control: 1.7 mm (N.S.) Horizontal bone change reentry: Test: (HTR): 1.9 mm, Control: 0.8 mm (P <0.05)
Wang et al. (1994)	Prospective split mouth RCT, 12 patients, 24 defects, 12 months	OFD +/- collagen membrane	PD change: Test (collagen): 2.84 mm, Control:1.92 mm (N.S.) CAL change: Test (collagen): 1.67 mm, Control: 0.67 mm (N.S.) Vertical bone fill change reentry: Test: 2.5 mm, Control: 1.5 mm (P <0.05)
Caton et al. (1994)	Prospective, parallel arm case-control clinical study, 40 patients, 6 months	OFD +/-GTR (Vicryl)	PD change: Test: 3.3 mm, Control: 0.3 mm (P <0.001) CAL change: Test better than controls (P <0.002) Vertical bone change reentry: Test better than controls (P<0.004) Horizontal bone change reentry: Test better than controls (P <0.03)
Polson et al. (1995)	Case series, 9 patients, 9 defects, 6 months	OFD + Biodegradeable GTR (Atrisorb)	PD reduction: 3.1 mm Vertical-CAL improvement: 3.3 mm Horizontal-CAL improvement: 3.0 mm
Polson et al. (1995)	Case series, 29 patients but 27 mandibular defects, 6 months, Multicenter	OFD + Biodegradeable GTR (Atrisorb)	PD reduction: 2.2 mm Vertical-CAL gain: 1.7 mm Horizontal-CAL gain: 2.5 mm
Hugoson et al. (1995)	RCT Split mouth. 38 patients, 76 defects, 12 months, Multicenter	OFD + e-PTFE (Gore) vs Biodegradable GTR (Guidor)	PD change: Test: (bioresorbable GTR): 2.0 mm, Control (ePTFE): 2.2 mm (N.S.) CAL-horizontal change: Test: 2.2 mm, Control: 1.4 mm (P <0.05) CAL-vertical change: Test: 0.8 mm, Control: 0.4 mm (N.S.)
Machtei et al. (1996)	Prospective clinical trial, 28 patients, 4 years, 35 defects	OFD +/- e-PTFE GTR	PD change (GTR): 3 mm improvement at year one but year 4 N.R. CAL change (GTR): N.R. Vertical AL (GTR): 1.05 mm (but unclear what year) Horizontal AL (GTR): 2.59 mm (but unclear what year)

Becker et al. (1996)	Prospective case series, 50 patients but 31 defects, 12 months Multicenter	OFD + bioabsorbable GTR (Resolut)	PD reduction: 2.5 mm CAL improvement: 2.1 mm Horizontal attachment level improvement: 1.8 mm
Yukna and Yukna (1996)	Prospective split mouth clinical study, 59 patients, 6 to 12 months (mean 11.1) Multicenter	Either OFD versus Collagen GTR or Collagen GTR versus ePTFE GTR	PD change: $Test_1$: (ePTFE): 1.2 mm, $Test_2$ (Collagen): 1.6 mm Control: 1.3 mm CAL change: $Test_1$: (ePTFE): 0.4 mm, $Test_2$ (Collagen): 0.9 mm Control: 0.4 mm Horizontal defect fill improvement (re-entry): $Test_1$: (ePTFE): 1.7 mm, $Test_2$ (Collagen): 1.85 mm Control: 1.1 mm
Bouchard et al. (1997)	Prospective parallel arm RCT, 30 patients, 12 months	OFD and resorbable polyglycolic-polylactic membrane (PGA/PLA) GTR vs. e-PTFE GTR	PD change: Test: (PGA/PLA): 2.1 mm, Control: 1.8 mm (N.S.) CAL change: Test: (PGA/PLA): 1.5 mm, Control: 1.2 mm (N.S.) Horizontal probing depth change: Test: (PGA/PLA): 2.5 mm, Control: 2.7 mm (N.S.)
Garrett et al. (1997)	Prospective parallel arm, clinical study (multicenter), 162 patients, 12 months	OFD +/- Biodegradeable GTR (Atrisorb)	PD change: Test: 2.3 mm, Control: 2.1 mm (N.S.) CAL change: Test: 2.0 mm, Control: 1.6 mm (N.S.) Horizontal attachment level change: Test: 2.1 mm, Control: 2.1 mm (N.S.)
Scott et al. (1997)	Prospective split mouth clinical study, 12 patients, 6 months	OFD + DFDBA and GTR: ePTFE versus Laminar bone membrane (LAMBONE)	PD change: Test: (LAMBONE): 0 mm, Control (ePTFE): 0.3 mm (N.S.) Vertical bone change reentry: Test: 1.2 mm, Control: 1.0 mm (N.S.) Horizontal bone change reentry: Test: 2.0 mm, Control: 2.2 mm (N.S.)
Sanz et al. (1997)	Case series, multicenter, 21 patients, but 10 mandibular degree II furcation defects, 12 Months	OFD + biodegradable membrane (Resolut)	PD reduction: 3.6 mm CAL gain: 2.4 mm Horizontal attachment level gain: 3.4 mm
dos Anjos et al. (1998)	Prospective split mouth clinical study, 15 patients, 6 months	OFD + GTR: ePTFE versus cellulose	PD change: Test: (Cellulose): 3.27 mm, Control (ePTFE): 2.87 mm (N.S.) CAL change: Test: 2.8 mm, Control: 2.53 mm (N.S.) Vertical bone change reentry: Test: 4.0 mm, Control: 3.0 mm (N.S.) Horizontal bone change reentry: Test: 2.93 mm, Control: 2.87 mm (N.S.)
Lekovic et al. (1998)	Prospective, split mouth, case control clinical study, 14 patients, 6 months	OFD and periosteal membrane (PM) ("GTR") vs coronally positioned flap (CPF)	PD reduction: PM: 3.66 mm, CPF: 3.07 mm (N.S.) CAL gain: PM: 2.71 mm, CPF: 2.41 mm (N.S.) Vertical bone change reentry: PM: 1.93 mm, CPF: 0.20 mm ($P \leq 0.001$) Horizontal bone change reentry: PM: 1.60 mm, CPF: 0.13 mm ($P \leq 0.001$)
De Leonardis et al. (1999)	Prospective, split mouth, case control clinical study, 12 patients, 12 months	OFD+ Bioabsorbable GTR (PLA, Guidor) +/- DFDBA	PD reduction: Test: (GTR + DFDBA): 2.8 mm, Control (GTR): 2.4 mm (N.S.) CAL gain: Test: 2.3 mm, Control: 2.0 mm (N.S.) Horizontal PD reduction: Test: 2.4 mm, Control: 1.8 mm ($P < 0.05$)

Karapataki et al.(1999)	Prospective, randomized, split mouth, case control clinical study, 11 patients with 11pairs of defects, 12 months	OFD + Test: GTR resorbable, (PLA, Guidor) vs. control GTR non-resorbable (ePTFE GoreTex)	CAL vertical gain: Test: 1.12 mm, Control: 0.5 mm (N.S.) CAL horizontal gain: Test: 1.8 mm, Control: 0.5 mm (P <0.05)
Simonpietri et al.(2000)	Prospective, split mouth, case control clinical study, 14 patients with 15 pairs of defects, 6 months	OFD + GTR (cellolose) +/- BHA	PD reduction: Test: (GTR+BHA): 1.93 mm, Control (GTR): 1.73 mm (N.S.) CAL gain: Test: 1.07 mm, Control: 1.27 mm (N.S.) Vertical bone gain reentry: Test: 1.8 mm, Control: 1.6 mm (N.S.) Horizontal bone gain reentry: Test: 2.47 mm, Control: 3.27 mm (P <0.05)
Yukna at al.(2001)	Prospective, split mouth, clinical study, 27 patients with 27 pairs of defects, 6 months	OFD + Bioactive glass (PG, PerioGlas) versus ePTFE GTR	PD reduction: Test: (Bioactive glass): 1.4 mm, Control (ePTFE): 1.1 mm (N.S.) CAL gain: Test: 0.4 mm, Control: 0.3 mm (N.S.) PD horizontal reduction: Test: 1.5 mm, Control: 1.2 mm (N.S.) Vertical bone gain reentry: Test: 1.1 mm, Control: 1.0 mm (N.S.) Horizontal bone gain reentry: Test: 1.4 mm, Control: 1.3 mm (N.S.)
Calongne et al. (2001)	Prospective, split mouth, clinical study, 8 patients with 24 defects, 6 months	OFD +/- HTR (PMMA + PHEMA + calcium hydroxide) +/- ePTFE GTR, Gore-Tex)	PD reduction: $Test_1$: (HTR): 1.6 mm, $Test_2$(ePTFE): 0.9 mm $Test_3$ (HTR+ePTFE): 1.3 mm (N.S.) CAL gain: $Test_1$: 1.4 mm, $Test_2$: 0.8 mm $Test_3$: 1.3 mm (N.S.) Vertical bone gain reentry: $Test_1$: 0.4 mm, $Test_2$: -0.4 mm $Test_3$: 0.1 mm (N.S.) Horizontal bone gain reentry: $Test_1$: 1.8 mm, $Test_2$: 0.7 mm $Test_3$: 1.1 mm (N.S.)
Pruthi et al. (2002)	Prospective, randomized, split mouth, clinical study, 17 patients with 36 defects, 12 months	OFD+ GTR (Collagen, CollaTec) vs GTR (e-PTFE, Gore-Tex)	PD reduction: e-PTFE: 1.12 mm, Collagen: 1.47 mm (N.S.) CAL vertical gain: e-PTFE: 0.47 mm, Collagen: 0.65 mm (N.S.) CAL horizontal gain: e-PTFE: 0.41 mm, Collagen: 0.41 mm (N.S.) Vertical bone gain reentry: e-PTFE: -1.0 mm, Collagen: 0.81 mm (P <0.05)
Prathibha et al. (2002)	Prospective, randomized, split mouth, clinical study, 10 patients with 20 defects, 6 months	OFD +/- GTR (Teflon, TefGen)	PD reduction: Test: (GTR): 1.95 mm, Control: 0.93 mm (N.R.) CAL vertical gain: Test: 1.66 mm, Control: 0.61 mm (P <0.05) Vertical bone change reentry: Test: 1.08 mm, Control: 0.16 mm (P <0.05) Horizontal bone change reentry: Test: 2.4 mm, Control: 0.64 mm (P <0.05)
Maragos et al. (2002)	Prospective, randomized, split mouth, clinical study, 17 patients with 36 defects, 12 months	3 groups: Calcium sulfate alone (C), Calcium sulfate+ doxycycline (CDX), Calcium sulfate + DFDBA (CDF)	PD vertical reduction: N.R except CDF 0.1 mm better reduction than C CAL gain: C: 1.4 mm, CDX: 1.9 mm, CDF: 2.7 mm ; CDF vs C; P <0.001, CDX vs C; P <0.001 PD horizontal reduction: CDF +CDX better reduction than C; P N.R. CAL horizontal: C: 0.7 mm, CDX: 1.3 mm, CDF: 2.1 mm; P N.R. Vertical bone change reentry: C: 2.8 mm, CDX: 3.1 mm, CDF: 1.9 mm; P N.R. Horizontal bone change reentry: C: 2.9 mm, CDX: 2.25 mm, CDF: 1.5 mm Data derived from figures thus not exact numbers.

Camargo et al. (2002)	Prospective parallel arm clinical study, 25 patients, 6 months	OFD + DFDBA + various concentrations of bovine derived bovine protein (BP)	PD change: Test: 1.0-1.8 mm, Control: 1.3 mm CAL change: Test: 0.5-1.5 mm, Control: 0.5 mm Horizontal Attachment level change: Test: 0.4-1.8 mm, Control: 1.9 mm *P*-values: N.R.
Cury et al. (2003)	Prospective, randomized, split mouth, clinical study, 9 patients with 18 defects, 6 months	OFD +/- GTR (Guidor)	PD reduction: Test: (GTR): 1.67 mm, Control: 2.51 mm (N.S.) CAL vertical gain: Test: 0.62 mm, Control: 1.16 mm (N.S.) CAL horizontal gain: Test: 2.27 mm, Control: 1.01 mm (P <0.05)
Camelo et al. (2003)	Case report, one patient, 9 months	OFD + rhPDGF-BB in DFDBA	PD reduction: 6 mm CAL vertical gain: 6 mm PD horizontal reduction: 4 mm Histology showed new cementum, PDL and bone
Lekovic et al. (2003)	Prospective, split mouth, clinical study, 26 patients with 52 defects, 6 months	OFD +/- (BHA (BioOss)+ PRP + GTR (BioGide)	PD change: Test: (BHA+PRP+GTR): 4.07 mm, Control: 2.49 mm (N.S.) CAL gain: Test: 3.29 mm, Control: 1.68 mm (N.S.) Vertical bone gain reentry: Test: 2.56 mm, Control: -0.19 mm (N.S.) Horizontal bone gain reentry: Test: 2.28 mm, Control: 0.08 mm (N.S.)
Donos et al. (2003)	Case series, 10 patients, with 12 teeth and 8 buccal (and 8 lingual furcations), 36 months	OFD + 24 % EDTA gel+ EMD	CAL gain vertical:* 0.8 mm CAL gain horizontal:*0.6 mm (*only buccal reported here)
Cury et al. (2003)	Prospective, randomized split mouth, clinical study, 9 patients with 18 defects, 24 months	OFD +/- GTR (Guidor)	PD reduction: Test: (GTR): 2 mm, Control: 1 mm (N.S.) CAL gain: Test: 0.45 mm, Control: 0.45 mm (N.S.) CAL gain horizontal: Test: 1.9 mm, Control: 1 mm (P <0.05) Vertical radiographic bone gain: Test: 1.3 mm, Control: 0.05 mm (P <0.05)
Jepsen et al. (2004)	Multicenter, prospective, randomized, split mouth, clinical study, 45 patients with 90 defects, 14 months	OFD + GTR (Resolut) versus EMD	PD reduction: See Meyle et al. 2004 CAL reduction: See Meyle et al. 2004 Horizontal bone gain reentry: Test: (EMD): 2.6 mm, Control (GTR): 1.9 mm (P <0.05)
Meyle et al. (2004)	Multicenter, prospective, randomized, split mouth, clinical study, 45 patients with 90 defects, 14 months	OFD + GTR (Resolut) versus EMD	PD reduction: Test: (EMD): 0.5 mm, Control (GTR): 0.25 mm CAL gain: Test: 0.5 mm, Control: 0.38 mm Horizontal bone change reentry: See Jepsen et al. 2004

Bremm et al. (2004)	Prospective, randomized, split mouth, clinical study, 10 patients with 20 defects, 6 months	OFD +/- GTR (Resolut)	PD reduction: Test: (GTR): 3.07 mm, Control: 2.17 mm (P <0.05) CAL vertical gain: Test: 2.39 mm, Control: 1.61 mm (N.S.) CAL horizontal gain: Test: 2.48 mm, Control: 2.10 mm (N.S.)
Akbay et al. (2005)	Prospective split mouth clinical study, 10 patients, 20 defects, 6 months	OFD +/- PDL graft taken after extraction of 3rd molar	PD reduction: Test: (PDL): 1.1 mm, Control: 1.6 mm (N.S.) CAL gain: Test: 0.8 mm, Control: 1.0 mm (N.S.) CAL horizontal: Test:1.3 mm, Control: 1.4 mm (N.S.) Vertical bone change reentry: Test: 0.8 mm, Control: 0.4 mm (P <0.05) Horizontal bone change reentry: Test: 2.175 mm, Control: 1.319 mm (P <0.01)
Belal et al. (2005)	Prospective randomized case-control clinical study, 20 patients, 50 defects, 12 months	1.GTR (PGA/PLA) 2.GTR (PGA/PLA + HA) 3. CT graft (CTG) + HA 4. OFD alone	PD reduction: 1: 3.43 mm, 2: 3.39 mm, 3: 3.23 mm, 4: 3.33 mm, 5 (control): 2.23 mm CAL vertical gain: 1: 3.34 mm, 2: 3.47 mm, 3: 3.07 mm, 4: 3.2 mm, 5 (control): 2.03 mm CAL horizontal gain: 1: 3.9 mm, 2: 4.5 mm, 3: 3.7 mm, 4: 4.5 mm, 5 (control): 2.3 mm
Tsao et al. (2006)	Prospective randomized case-control clinical study, 27 patients, 6 months	OFD alone or OFD + mineralized bone allograft (MBA, Puros) +/- collagen GTR (Biomend)	PD reduction: $Test_1$ (MBA+GTR): 0.7 mm, $Test_2$ (MDA alone): 0.9 mm* Control (OFD): 0.1 mm CAL gain: $Test_1$: 0.3 mm, $Test_2$: -0.1 mm, Control: 0.9 mm PD horizontal reduction: $Test_1$: 1.1 mm*, $Test_2$: 1.2 mm, Control: 0.9 mm Vertical bone gain reentry: $Test_1$: 0.2 mm, $Test_2$: 0.7 mm* Control: 0.0 mm Horizontal bone gain reentry: $Test_1$: 1.1 mm*, $Test_2$: 1.1 mm*, Control: 0.2 mm (*P<0.05)
Chitsazi et al. (2007)	Prospective randomized split mouth clinical study, 10 patients, 6 months	OFD +/- EMD	PD reduction: Test: (EMD): 1.95 mm, Control: 1.55 mm (N.S.) CAL gain: Test: 1.45 mm, Control: 0.9 mm (N.S.) CAL horizontal: Test: 1.9 mm, Control: 0.6 mm (P <0.01) Vertical bone change reentry: Test: 1.25 mm, Control: 0.85 mm (P<0.05) Horizontal bone change reentry: Test: 2.0 mm, Control: 0.8 mm (P <0.01) (N.S.)
Eto et al. (2007)	Prospective randomized split mouth clinical study, 10 patients, 6-7 months	OFD +/- BHA (P-15, PepGen)	PD reduction: Test: (BHA + P-15): 0.8 mm, Control (OFD alone): 1.6 mm (N.S.) CAL gain vertical: Test: 1.7 mm, Control: 2.1 mm (N.S.) CAL gain horizontal: Test: 2.4 mm, Control: 1.5 mm (N.S.) Radiographic bone gain: Test: 0.9 mm, Control: 0.6 mm (N.S.)
Aimetti et al.(2007)	Case series, 11 patients, 24 months	EMD + autologous bone	PD reduction: 1.67 mm CAL gain vertical: 2.23 mm Bone sounding reduction horizontal: 3.39 Bone sounding reduction vertical: 3.64 mm

Lyons et al. (2008)	Prospective randomized case-control clinical study, 29 patients, 9 months	OFD + DFDBA + Polylactic acid (PLA) GTR +/- Doxycycline (PDox) or DFDBA alone	PD reduction: $Test_1$ (DFDBA + PLA + PDox): 1.55 mm, $Test_2$ (DFDBA + PLA - PDox): 1.9 mm Control (DFDBA alone): 1.27 mm (N.S.) CAL gain: $Test_1$: 0.22 mm, $Test_2$: 1.3 mm, Control: 1.9 mm (N.S.) PD horizontal reduction: $Test_1$: 1.22 mm, $Test_2$: 1.3 mm, Control: 0.6 mm (N.S.) Vertical bone gain reentry: $Test_1$: 1.4 mm, $Test_2$: 1.11 mm Control: 2.0 mm (N.S.) Horizontal bone gain reentry: $Test_1$: 2.33 mm, $Test_2$: 2.11 mm, Control: mm (N.S.)
Kothiwale et al. (2009)	Prospective randomized split mouth clinical study, 10 patients, 9 months	OFD and (BHA) Bio-Oss + Amnionic (AM) GTR or DFDBA + Amnionic GTR	PD reduction: Test (DFDBA +AM): 4.7 mm, Control (BHA +AM): 4.4 mm (N.S.) CAL gain: Test: 4.8 mm, Control: 5.1 mm (N.S.) Radiographic bone gain: Test: 0.57 mm, Control: 0.77 mm (P<0.05)
Pradeep et al. (2009)	Prospective randomized split mouth clinical study, 20 patients, 6 months	OFD +/- PRP	PD reduction: Test: (PRP): 2.3 mm, Control: 0.8 mm (P <0.05) CAL gain: Test: 2.5 mm, Control: 0.1 mm (P <0.01) CAL horizontal gain: Test: 2.5 mm, Control: 0.8 mm (P <0.001) CT: Vertical: Test: 1.23 mm, Control: 0.64 mm (P <0.05) Horizontal: Test: 1.33 mm, Control: 0.09 mm (P <0.01)
Taheri et al. (2009)	Prospective randomized case control clinical study, 14 patients, 18 furcations	OFD +BHA (Bio-Oss) +/- Collagen GTR (BioGide)	PD reduction: Test: (BHA+ GTR): 2.4 mm, Control: 2.4 mm (N.S.) CAL gain: Test: 2.1 mm, Control: 1.9 mm (N.S.) CAL horizontal: Test: 1.2 mm, Control: 1.6 mm (N.S.) Vertical bone gain reentry: Test: 2.1 mm, Control: 1.9 mm (N.S.) Horizontal bone change reentry: Test: 2.4 mm, Control: 2.1 mm (N.S.)
Santana at al. (2009)	Prospective case control clinical study, 60 patients, 12 months	OFD +/- microgranular HA mixed with tetracycline	PD reduction: Test: (HA+Tetracycline): 3.65 mm, Control: 0.60 mm (P <0.05) CAL vertical gain: Test: 3.05 mm, Control: 0.65 mm (P <0.05) CAL horizontal gain: Test: 3.45 mm, Control: 0.55 mm (P <0.05)
Pradeep et al. (2011)	Prospective randomized split mouth clinical study, 18 patients, 9 months	OFD +/- PRF clot and PRF membrane	PD reduction: Test: (PRF): 4.06mm, Control: 2.89 mm (P <0.01) CAL vertical gain: Test: 2.33 mm, Control: 1.28 mm (P <0.01) CAL horizontal gain: Test: 2.67 mm, Control: 1.89 mm (P <0.01)

Appendix III. Clinical trials on regenerative treatment of mandibular molar buccal degree II furcation defects. Pubmed database were searched with the following terms and key words and limited to clinical trials: furcation and treatment (total hits 166) until the date 06.04.2011. Furthermore the reference lists in the publications listed were manually searched for additional references. Studies mixing mandibular and maxillary degree II furcation defects excluded if not results for the mandibular molars were reported separately. Results reported as mean values. N.R.= Not reported, N.S. Non significant difference between groups, RCT= Randomized clinical trial

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Appendix IV

Appendix IV

Description of markers analyzed in paper I

Wound fluid analyses

To study the progress of cortical bone healing after 4 weeks of healing of sham PTG and heat oxidized PTG (WPTG) treated defects, lactate dehydrogenase (LDH) activity, bone alkaline phosphatase (ALP) activity and total protein in the wound fluid was evaluated.

ALP is a membrane bound glycoprotein produced by osteoblasts and attached to the outer cell surface of osteoblasts. It is required for osteoid formation and extracellular matrix mineralization.[1] It is thus a good indicator of bone healing and primary mineralization.
LDH release from cells is an index of tissue necrosis.[2] LDH is a cytoplasmatic protein. At cell death the cellmembanes are opened and LDH will be released to the extracellular environment.
Total protein is measured for achieving values to be used for adjusting other measured specific proteins or their total activity. Protein concentrations are usually determined and reported with reference to standards of a common protein e.g., bovine serum albumin (BSA), and are used to correct other measured specific proteins (by ELISA, for example) or their activity. Total protein in the sample can consequently be used to correct that all samples have equal quantity.[3]

Markers analyzed by RT-PCR

A number of phenotypic markers are characteristic of differentiated osteoblasts like osteocalcin (OCN) and runt-related transcription factor-2 (runx2) and osteoclasts like tartrate-resistant acid phosphatase **(**TRAP) and H^+ adenosine triphosphate **(**ATP)-ase. The detection

of their gene expression levels gives an indication of osteoclast or osteoblast activity at the bone-implant interface

OCN which is also called bone γ-carboxyglutamic acid (Gla protein or BGP) is an osteoblast-specific protein and a major non-collagenous matrix protein of bone.[4] It is synthesized by osteoblasts and constitues 1-2 % of the total protein in bone.[5]

Collagen type I **(Coll-I)** accounts for 90 % of the total protein fraction in bone. It is abundant in peripheral tissues and it has been demonstrated that coll I signaling reduces receptor activator of nuclear factor κβ ligand (RANKL) expression from T-cells which to the least potentially may lead to a lower ability of these cells to induce osteoclast formation.[6, 7]

Runx2 is a member of the runt homology domain family of transcription factors, which are essential for osteoblast differentiation and a key regulator of bone formation. Moreover Runx2 regulate some proteins (e.g., matrix metalloproteinase (MMP)-9[8]) which again regulates vascular invasion in bone and cell migration.[8-10]

TRAP is expressed by osteoclasts and thus a good marker for bone resorption.[11]

Vacuolar H^+-ATPase is involved in bone resorption and resides on the membrane of osteoclasts. Vacuolar H^+-ATPase is pumping H+ from the cytoplasm and is thus mediating the acidification of the extracellular environment in the resorption lacuna.[12]

The detection of the pro-inflammatory cytokines, TNF-α and IL-6 was used to evaluate macrophage activation. Tumor necrosis factor -α (TNF-α) and interleukin **(**IL) -6 are promoters of osteoclast differentiation and cytokine secretion.

IL-6 is a pro-inflammatory cytokine, which was evaluated in the study to determine macrophage activation. IL-6 is a pleiotropic (i.e., multifunctional) molecule which is secreted by monocytes, macrophages, T-helper cells and bone-marrow stromal cells and promote innate (i.e., non-specific immunity and the "first line of defense to pathogens") immunity and elimination of pathogens. It promotes terminal differentiation of B-cells into plasma cells and stimulate antibody secretion.[13]

TNF-α is a pro-inflammatory cytokine, which was used to evaluate macrophage activation. TNF-α is secreted by macrophages and mast cells and promotes innate immunity and elimination of pathogens.[13] The effect of TNF-α on osteoblastogenesis is blockage of osteoblast differentiation by inhibition of two transcription factors indispensable for bone formation: Runx2 and Osterix (Osx).[14]

IL-10 is secreted by T helper cells, targets macrophages and antigen presenting cells and suppresses cytokine production. IL-10 inhibit both inflammation and osteoclastogenesis.[13]

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